Universidad de Navarra

Facultad de Ciencias

Papel de la proteína fosfatasa 2A (PP2A) y su inhibidor endógeno SET en la leucemia mieloide aguda

Ion Cristóbal Yoldi

Tesis doctoral

2011

Servicio de Publicaciones de la Universidad de Navarra

ISBN 978-84-8081-309-9

Universidad de Navarra

Facultad de Ciencias

Papel de la proteína fosfatasa 2A (PP2A) y su inhibidor endógeno SET en la leucemia mieloide aguda

Memoria presentada por D. Ion Cristóbal Yoldi para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Genética y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 4 de Marzo de 2011

Dra. María Dolores Odero de Dios

AGRADECIMIENTOS

En primer lugar me gustaría dar las gracias a mis padres, por la enorme paciencia que habéis tenido conmigo y por ser un apoyo fundamental siempre, menos mal que sólo tenéis un hijo porque os da guerra como cinco o seis. Y como no a mis abuelos, por estar cada día conmigo y por haberme acompañado de cerca en todos los acontecimientos de mi vida. Gracias también al resto de mi familia por preocuparos siempre por mí. En fin, sólo espero que podáis estar orgullosos de mí como yo lo estoy de vosotros, y ya sabéis lo mucho que os quiero y necesito.

Una persona a la que me gustaría expresar mi agradecimiento de forma especial es a Lola, mi directora de tesis, una jefa comprensiva y una persona estupenda. Gracias por haberme dado la oportunidad de trabajar estos años contigo, por haberme dejado desarrollar mis ideas, y por el apoyo a nivel personal que siempre he recibido de ti. Espero haber estado a la altura como doctorando y que estés satisfecha por el trabajo que hemos hecho.

Mil millones de gracias a ti, Laura, gracias por ser como eres, por estar a mi lado y por todo el cariño y ayuda que me has dado desde el primer momento que te conocí. Eres de esas personas que uno desea poder tener toda la vida a su lado y ten por seguro que sin ti no hubiese llegado hasta aquí. Rubén, Fermín, Ruiz, Retegui, Aitor, JM, Leti, Javi y otros buenos amigos míos que aunque poco saben de leucemias quien más o quien menos todos han servido de desahogo para mi en muchos momentos en los que me flaqueaban las fuerzas. Gracias señor Raúl C. por todas esas noches de jueves y mañanas de viernes inolvidables por distintos motivos, y porque contigo he ganado un amigo de los de verdad. Mikel G., hay que ver cómo pasa el tiempo, parece ayer cuando organizamos esa primera cena de primero de carrera y ahora acabaremos a la vez la tesis, un placer haber recorrido este camino a la vez que tú. Por supuesto no me olvido de mi gran amiga Paula y de nuestros almuerzos juntos, si no es por ti aún seguiría intentando hacer solo ese primer FISH que tanto marcó el devenir de mi tesis. Que sepas que eres genial y que eres muy importante para mí. Espero tenerte siempre cerca.

Cristina, no se que hubiese hecho sin tu ayuda, me alegro de todos los momentos que hemos pasado dentro y fuera del laboratorio, que sepas que en poco tiempo te he cogido un gran cariño y que me parece que vales mucho. Nerea M., gracias porque me enseñaste cuando no tenía ni idea de nada y siempre te has portado bien conmigo. Xabi y Oskar, mucho ánimo con vuestras tesis y que sepáis que aquí estoy para lo que necesitéis. Ruth, Leyre, Choni, Miren, Nerea B., siempre he podido recurrir a vosotros y me alegra haberos tenido de compañeras. Os deseo mucha suerte para el futuro. Y, por supuesto, María G.B., Iria y Carmen, gracias por ponérmelo tan fácil desde el primer día, por enseñarme tanto y por ser simplemente así, sin vosotras tengo claro que no hubiera sido lo mismo. Dani, Axel, Nuria, Kotryna, José R., Eloy, Igor, Jorge, Iker, Diego, Pedro, Nerea S, Guillermo, Maite M., Lorea, Patricia, Maite, Amaya y Patricia R., me alegro mucho de haberos conocido y os deseo lo mejor. Marta, has llegado casi al final de mi etapa pero tus consejos me han servido mucho y espero que sigamos en contacto. Tampoco me puedo olvidar de Eva, Marisol, Inés y Valentina, que me han ayudado siempre que han tenido la oportunidad y han sido un apoyo

fundamental para mí en los momentos difíciles en los que hubiera tirado la toalla, sois buenas científicas pero sobre todo un ejemplo a seguir como personas.

Algo que tendré siempre grabado es mi paso por el CIB en Madrid, en el laboratorio de Carmelo Bernabeu. Una persona fantástica que me acogió de la mejor forma posible y que ha sabido formar un grupo de gente estupenda. Blanco, gracias por enseñar tantas cosas a ese estudiante tan verde que te endosaron. A Luisa y Carmen por vuestra gran amabilidad. A Raquel, por muchos momentos inolvidables. A Rodrigo, por ser mi mejor compañero de cañas. África y Eva, por tratarme tan bien y compartir buenos ratos conmigo. Y a tantos otros compañeros porque entre todos hacíais una piña y siempre me hicisteis sentir parte de ella. Nunca os olvidaré.

Por último, me gustaría expresar mi sincero agradecimiento al Departamento de Genética de la Universidad de Navarra, del cual formo parte. María José Calasanz, no se qué hubiera hecho sin tus muestras, muchas gracias por tu ayuda y por tu tiempo. José Luis Vizmanos, gracias de corazón por tu amabilidad y por tus consejos a lo largo de este tiempo. Marina García Delgado, no me olvido que fue gracias a ti que entré a formar parte del Departamento, como alumno me ayudaste al final de mi carrera en un momento complicado y nunca te lo agradeceré bastante. Javier Novo, fuiste un gran profesor y me ayudaste mucho al principio del camino. Al resto de gente del Departamento, gracias porque sólo tengo buenas palabras para todos/as. Y también quisiera mencionar a Luis Montuenga, una gran persona con la que siempre he podido contar y que se ha preocupado por seguir mi tesis de cerca, muchas gracias Luis.

En general, a todos los compañeros del CIMA y de la Universidad de Navarra que han compartido conmigo estos años, os estoy muy agradecido porque todos habéis puesto vuestro granito de arena en esta experiencia maravillosa. Siento que he crecido mucho tanto a nivel personal como profesional y eso es culpa de la suma de todos los momentos que he vivido con cada uno de vosotros. Gracias por haberme hecho llegar al final y un abrazo de todo corazón.

ABREVIATURAS

AML	Acute myeloid leukemia
BAC	Bacterial artificial chromosome
BM	Bone marrow
ChIP	Chromatin immunoprecipitation
CIP2A	Cancer inhibitor of PP2A
CML	Chronic myeloid leukemia
CR	Complete remission
DNA	Deoxyribonucleic acid
DFS	Disease-free survival
DR	Downregulation
EFS	Event-free survival
FISH	Fluorescence in situ hybridization
ITD	Internal tandem duplication
LOH	Loss of heterozigosity
MAPK	Mitogen activated protein kinase
Mb	Megabases
MDS	Myelodysplastic syndrome
NK	Normal karyotype
OE	Overexpression
OS	Overall survival
PB	Peripheral blood
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
QRT-PCR	Quantitative reverse transcriptase-polimerase chain reaction
RNA	Rybonucleic acid
sAML	Secondary acute myeloid leukemia
shRNA	Short hairping RNA
siRNA	Short interfering RNA
WHO	World health organization

ÍNDICE

INTRODUCCIÓN

La leucemia mieloide aguda (LMA) es una enfermedad clínica y molecularmente heterogénea, que surge como consecuencia de alteraciones genéticas y epigenéticas adquiridas en células progenitoras hematopoyéticas. Se caracteriza por una proliferación incontrolada de células inmaduras llamadas blastos, que infiltran la médula ósea e invaden la sangre periférica y otros órganos. Aunque su etiología es aún desconocida, la LMA, como otros tipos de cáncer, tiene un origen clonal y es la consecuencia de una progresiva acumulación de mutaciones (Estey et al., 2006; Deschler et al., 2006). Este tipo de leucemia presenta una prevalencia de 3,8 casos por cada 100.000 habitantes, aumentando hasta 18 casos por cada 100.000 habitantes en la población mayor de 65 años. Por tanto, es una enfermedad que afecta preferentemente a adultos. Tres de cada 5 afectados son varones.

A pesar de los avances realizados en el conocimiento de la biología de esta enfermedad y del desarrollo de nuevas terapias, el porcentaje de pacientes que recaen continúa siendo elevado (Rowe, 2009) (Figura 1). La mediana de edad de los pacientes con LMA es superior a los 65 años; sin embargo, los avances en el tratamiento de esta enfermedad se limitan al grupo de pacientes menores de 60 años, a pesar de que los mayores de 60 años representan al menos dos tercios del total de los casos (Buchner et al., 2009). Más del 70% de los pacientes menores de 60 años alcanza remisión completa tras combinaciones de tratamientos con quimioterapia basada en antraciclina. El tratamiento de consolidación con altas dosis de citarabina o el transplante en pacientes de alto riesgo, reduce la tasa de recaídas a aproximadamente 50% de los casos. En los pacientes mayores de 60 años, la primera decisión es sobre la intensidad del tratamiento que pueden recibir. En los pocos casos en los que se pueden aplicar tratamientos similares basados en antraciclina/citarabina, la tasa de remisión es del 50%, aunque el 80% recae a los 3 años. Sin embargo, en los pacientes mayores de 60 años que no son susceptibles de recibir una quimioterapia intensa, la supervivencia se reduce a sólo 4 meses, por lo que resulta de gran interés el desarrollo de alternativas terapéuticas para tratar a este grupo de pacientes (revisado en Burnett et al., 2011). Por lo tanto, es importante tanto la identificación de marcadores genéticos con valor pronóstico como el desarrollo de nuevas estrategias terapéuticas en LMA (Döhner et al., 2010).

En los últimos años, el análisis genético ha aportado una información indispensable para el diagnóstico, pronóstico y tratamiento de este tipo de leucemia. El cariotipo es en la actualidad el factor pronóstico independiente más importante (Grimwade et al., 2009) (Figura 1), pero en la nueva clasificación WHO ya se han añadido alteraciones moleculares que definen subgrupos con distinto pronóstico (Vardiman, 2010). Sin embargo, en la actualidad, sólo el análisis de mutaciones en *NPM1*, *CEBPA* y *FLT3* ha sido incluido en la práctica clínica y afecta al diagnóstico, evaluación del riesgo y terapia. De hecho, en base a sus características clínicas y patológicas, la LMA con mutaciones en *NPM1* y la LMA con mutaciones en *CEBPA* fueron incorporadas en 2008 como entidades provisionales por la WHO. Por lo que respecta a las mutaciones de *FLT3*,

aunque no se considera que definan una entidad distinta de LMA por sí mismas, proporcionan una información pronóstica muy importante y son diana de inhibidores tirosina quinasa específicos que actualmente están en ensayos clínicos. Por tanto, se recomienda tanto en ensayos clínicos como en la práctica rutinaria analizar la presencia de mutaciones en *NPM1*, *CEBPA* y *FLT3*, al menos en aquellos pacientes que por tener cariotipo normal reciben como tratamiento de rutina quimioterapia en bajas dosis y tratamiento de soporte (revisado en Marcucci et al., 2011a).

La identificación de nuevas alteraciones genéticas ha permitido la identificación de pacientes que requieren un tratamiento específico, como es el caso de los pacientes con leucemia promielocítica aguda, que presentan una alteración específica, la t(15;17), que da lugar al gen de fusión *PML-RARA*. El tratamiento de estos pacientes con una molécula diana para esta alteración, el ácido trans-retinoico, que produce la diferenciación terminal de las células leucémicas, ha resultado en una tasa de remisiones de más del 90%, con una elevada supervivencia libre de enfermedad y una escasa toxicidad (Sanz et al., 2008; Sanz et al., 2009) (Figura 1). Por lo tanto, una mejor caracterización de las alteraciones genéticas en LMA es importante para definir con más precisión el pronóstico de cada paciente y desarrollar nuevas terapias dirigidas contra las dianas moleculares que participan en la trasformación leucémica.

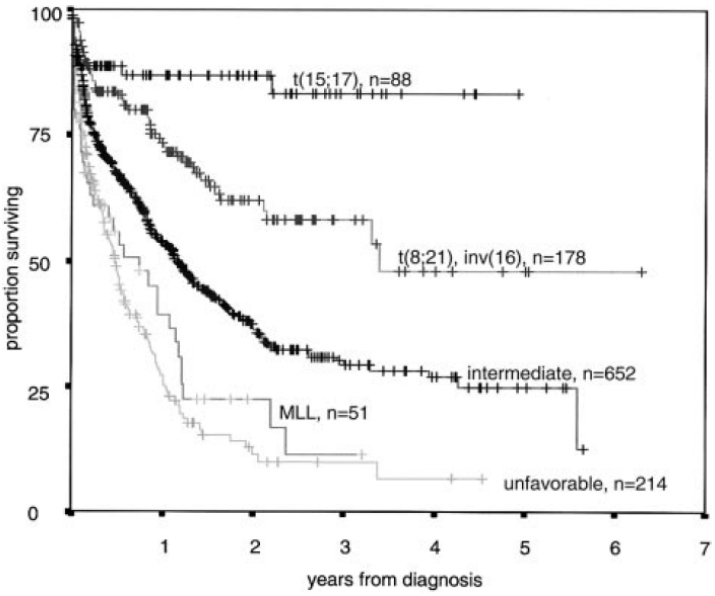


Figura 1. Supervivencia global en una serie de 1183 pacientes con LMA según criterios citogenéticos (Schoch et al., 2003).

Cada vez hay más evidencia de que en la génesis de la LMA intervienen tanto reordenaciones de genes que codifican factores de transcripción importantes en la hematopoyesis, como mutaciones de genes tirosina quinasas, de forma que se habla de la necesidad de una cooperación entre ambos sucesos para la génesis de la LMA, que produciría un aumento de la proliferación y/o una mayor supervivencia, junto con una detención de la diferenciación. En los últimos años, sin embargo, el descubrimiento de la desregulación de varios microRNAs con relevancia funcional ha añadido un mayor nivel de complejidad al entendimiento del proceso de desarrollo de la LMA. De hecho, se han identificado grupos de

microRNAs con valor pronóstico e incluso se ha descrito que un solo microRNA (miR-181a) puede proporcionar información pronóstica complementaria a la obtenida por la citogenética, y por la presencia de mutaciones o alteraciones en la expresión de determinados genes (revisado en Marcucci et al., 2011b).

La fosforilación reversible es uno de los mecanismos por los que la célula mantiene un balance correcto entre señales inductoras y supresoras tumorales. De este modo, el equilibrio entre quinasas y fosfatasas es esencial para el control de una correcta proliferación, apoptosis y diferenciación (Hunter, 1995). Hay muchos estudios que analizan el comportamiento aberrante de las proteínas quinasas en cáncer. Uno de los mejores ejemplos es la oncoproteína BCR-ABL en la leucemia mieloide crónica (LMC) y leucemia linfoblástica aguda (LLA) Philadelphia positiva (Westbrook et al., 1992; Calabretta et al., 2004). También se ha demostrado el papel importante que juegan las alteraciones de genes tirosina quinasas como *FLT3* ó *JAK2* en el desarrollo de la LMA; incluso se están realizando en la actualidad ensayos clínicos para evaluar el potencial terapéutico de varios compuestos inhibidores de FLT3 (Levis, 2010; Ferrajoli et al., 2007; Quintás-Cardama et al., 2010a; Quintás-Cardama et al., 2010b). Sin embargo, hay pocos estudios acerca del papel de las proteínas fosfatasas en cáncer, a pesar de que juegan un papel clave en el control de la señalización celular.

Numerosos trabajos en los últimos años se han centrado en la proteína fosfatasa 2A (PP2A), una proteína supresora tumoral que ejerce una función reguladora central en la célula, controlando gran variedad de funciones y rutas de señalización. La inactivación de PP2A parece ser un evento esencial en diversos tipos de cáncer, de modo que podría tratarse de una alteración muy inicial y común en el proceso de transformación maligna y por tanto, una diana con un enorme potencial en terapias dirigidas a su activación.

Proteína fosfatasa 2A (PP2A)

El término proteína fosfatasa 2A (PP2A) no hace referencia a una única proteína sino a una larga colección de enzimas oligoméricas con actividad serina-treonina fosfatasa que junto con la proteína fosfatasa 1 (PP1) suponen en torno al 90% de la de actividad serina-treonina fosfatasa presente en células eucariotas (Millward et al., 1999).

1. Estructura y función

El núcleo enzimático de PP2A está compuesto por una subunidad A estructural (también conocida como PR65 ó PPP2R1) y una subunidad C catalítica (también llamada PP2Ac ó PPP2C), que dan lugar a un heterodímero A-C (Figura 2). En mamíferos, tanto la subunidad catalítica como la estructural están codificadas por 2 genes diferentes, dando lugar a las isoformas α y β de cada una de ellas:

- Subunidades estructurales: Aα (PR65α/PPP2R1A) y Aβ (PR65β/PPP2R1B) (Hemmings et al., 1990).
- Subunidades catalíticas: Cα (PP2Ac-α/PPP2CA) y Cβ (PP2Ac-β/PPP2CB) (Stone et al., 1987).

A este heterodímero A-C se une una tercera subunidad B reguladora cuya función consiste en determinar tanto la especificidad de substrato del complejo enzimático

como su localización intracelular. Hasta el momento se han identificado 4 familias de subunidades B reguladoras:

- B/B55/PR55/PPP2R2 (Mayer et al., 1991; Zolnierowicz et al., 1994; Strack et al., 1999; Li et al., 2002).
- B´/B56/PR61/PPP2R5 (McCright et al., 1995; Csortos et al., 1996; Tehrani et al., 1996).
- B´´/PR72/PPP2R3 (Hendrix et al., 1993; Yan et al., 2000; Stevens et al., 2003).
- B´´´/PR93/SG2NA/PR110/Striatin (Moreno et al., 2000).

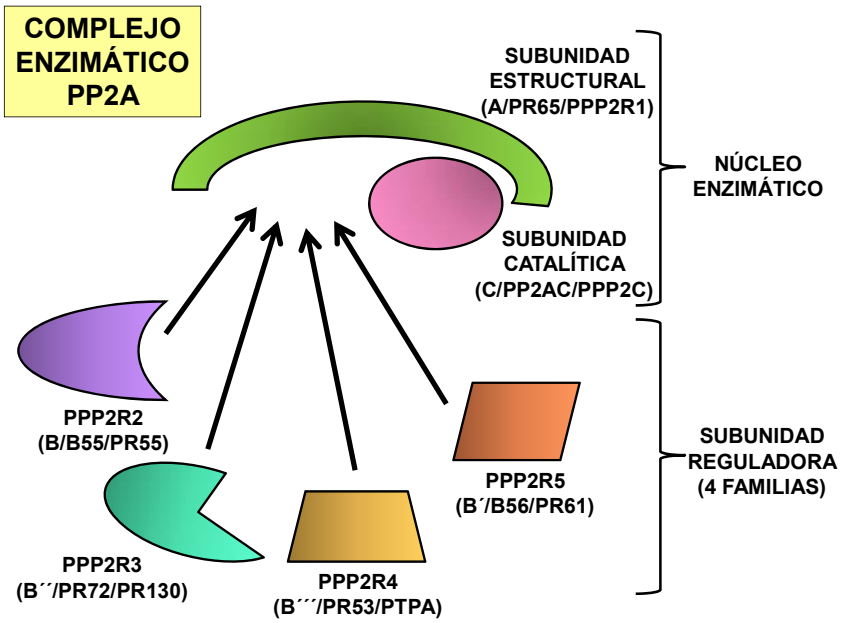


Figura 2. Estructura del heterotrímero PP2A mostrando la gran variedad de isoformas existentes para cada una de las subunidades A, B y C (adaptada de Westermarck y Hahn, 2008).

1.1. Subunidad estructural (A/PR65/PPP2R1)

La subunidad A estructural está formada por 15 repeticiones Huntington/Elongation/A-subunit/TOR (HEAT) en tándem, a través de las que interacciona con las subunidades B y C. Estudios estructurales han demostrado que las subunidades B reguladoras interaccionan con los dominios HEAT 2-8, situados en el extremo amino-terminal de la subunidad A estructural, mientras que la subunidad C catalítica se une a través de los HEAT 11-15, en el extremo carboxilo-terminal (Groves at al, 1999; Xu et al., 2006; Cho et al., 2007).

El papel que juega la subunidad A es de carácter estructural y se ha visto que alteraciones en un solo aminoácido tanto en la isoforma Aα como Aβ son capaces de interrumpir su interacción tanto con la subunidad C catalítica como con subunidades B específicas (Ruediger et al., 2001, a; Ruediger et al., 2001, b; Chen et al., 2005; Esplin et al., 2006; Sablina et al., 2007), lo cual sugiere que las subunidades A estructurales son fundamentales para la composición de los complejos PP2A (Ruediger et al., 1999). Además, diversos estudios tridimensionales han mostrado la gran flexibilidad estructural de la subunidad A,

que sufre cambios conformacionales tras la interacción con las subunidades B y C, lo que aporta al complejo PP2A una capacidad de adaptación adicional en el proceso de reconocimiento de substrato, de modo que éste quede accesible a la subunidad catalítica (Xing et al., 2006; Xu et al., 2006; Cho et al., 2007).

La mayor parte de los complejos PP2A contienen la isoforma Aα, mientras que sólo una pequeña fracción (~10%) incluye la Aβ. Sólo en fases iniciales del desarrollo se presenta Aβ con más abundancia que Aα (Bosch et al., 1995). Por otra parte, aunque ambas isoformas comparten un 87% en identidad de secuencia, difieren en su capacidad de interacción con subunidades B reguladoras (Zhou et al., 2003).

Se ha propuesto que la formación del complejo PP2A activo requiere la acción de la peptidil-prolil-isomerasa PTPA, que se une a la subunidad A y actúa como activador de un comp lejo PP2A inicialmente inactivo mediante un mecanismo de acción que todavía no se conoce, pero que podría incluir la inhibición de la metilesterasa PME-1 (Longin et al., 2004). La proteína PME-1, además de inhibir el reclutamiento de subunidades PPP2R2, en ausencia de subunidad A, es capaz de inhibir la formación de una subunidad C activa, lo que sugiere que puede ejercer alguna función en la activación inicial del complejo PP2A (Hombauer et al., 2007).

1.2. Subunidad catalítica (C/PP2Ac/PPP2C)

La subunidad C catalítica incluye un sitio activo que tiene como diana grupos fosfato situados en serinas o treoninas, y sólo en algunos casos es capaz de actuar sobre tirosinas fosforiladas (Fellner et al., 2003). De las dos isoformas existentes, Cα se expresa en niveles unas 10 veces mayores que Cβ, ya que ésta se encuentra bajo la acción de un promotor de menor actividad (Khew-Goodall et al., 1988). Ambas isoformas tienen un peso molecular de 35 kDa y un 97% de identidad de secuencia, y presentan una gran regulación a nivel transcripcional, lo que dificulta su expresión ectópica (Baharians et al., 1998). Además, la pérdida de Cα es letal en ratones (Gotz et al., 1998), lo que indica su enorme importancia.

La subunidad C presenta un tallo carboxilo-terminal muy conservado ($_{304}$TPDYFL$_{309}$) cuyo patrón de metilación y fosforilación va a determinar el reclutamiento de determinadas subunidades B reguladoras al heterodímero A-C. Así, la metilación del residuo L309 por LCMT1 es necesaria para la unión de miembros de la familia PPP2R2, pero no para otras subunidades B (Longin et al., 2007). Además, esta metilación es reversible por la acción de PME-1 (Ogris et al., 1999). La fosforilación en la Y307 inhibe el reclutamiento de la familia PPP2R2, y también de PPP2R5A, PPP2R5B y PPP2R5E al núcleo enzimático A-C (Chen et al., 1992). Una de las proteínas que regula la fosforilación en Y307 es la quinasa c-SRC, lo que sugiere que la inhibición de las subunidades B es una parte del mecanismo de transformación inducida por este oncogén (Longin et al., 2007). Por último, se ha visto que la fosforilación en T304 es capaz de inhibir la unión de PPP2R2, pero no de otras subunidades B (Longin et al., 2007). El estado de metilación y fosforilación de estos residuos no parece influir en el reclutamiento del resto de subunidades B.

Por otra parte, la subunidad C comparte homología a nivel de secuencia con otras serina-treonina fosfatasas como la PP1 y la proteína fosfatasa 2B (PP2B ó

calcineurina) (Janssens et al., 2001). Además, se ha descrito que la interacción de la subunidad C con la proteína Tap42/a4 (Murata et al., 1997) da lugar a un heterodímero capaz de reprimir la apoptosis a través de la inhibición de c-Jun y p53 (Kong et al., 2004).

1.3. Subunidades B reguladoras

Con respecto a las subunidades B reguladoras, hasta el momento se han identificado 12 genes diferentes que codifican al menos 23 transcritos alternativos (Tabla 1). Mientras que la subunidad estructural Aα puede interaccionar con todas las subunidades B, la Aβ no interacciona con subunidades reguladoras de la familia PPP2R2 y se une preferentemente a miembros de la familia PPP2R3 (Zhou et al., 2003).

Tabla1. Relación de las subunidades del complejo PP2A (adaptada de Eichhorn et al., 2009).

NOMENCLATURA PRINCIPAL	NOMBRES ALTERNATIVOS	CÓDIGO ENSEMBL
Subunidad catalítica		
PPP2CA	**Cα / PP2Acα**	**ENST00000231504**
PPP2CB	**Cβ / PP2Acβ**	**ENST00000221138**
Subunidad estructural		
PPP2R1A	**Aα / PR65α**	**ENST00000322088**
		ENST00000361107
PPP2R1B	**Aβ / PR65β**	**ENST00000341980**
		ENST00000311129
Subunidad reguladora		
PPP2R2A	**Bα / PR55α**	**ENST00000315985**
PPP2R2B	**Bβ / PR55β**	**ENST00000287031**
		ENST00000336640
		ENST00000355212
PPP2R2C	**Bγ / PR55γ**	**ENST00000264959**
		ENST00000335585
PPP2R2D	**Bδ / PR55δ**	**ENST00000314348**
PPP2R3A	**B´´α / PR130**	**ENST00000264977**
PPP2R3B	**B´´β / PR72**	**ENST00000334546**
	PR70/48	**ENST00000300846**
		ENST00000361450
PPP2R4	**B´´´ / PR53 / PTPA**	**ENST00000337738**
		ENST00000266101
		ENST00000347048
		ENST00000357197
PPP2R5A	**B´α / PR61α**	**ENST00000261461**
PPP2R5B	**B´β / PR61β**	**ENST00000164133**
PPP2R5C	**B´γ1 / PR61γ1**	**ENST00000334743**
	B´γ2 / PR61γ2	**ENST00000334756**
	B´γ3 / PR61γ3	**ENST00000350249**
PPP2R5D	**B´δ / PR61δ**	**ENST00000230402**
		ENST00000344268
PPP2R5E	**B´ε / PR61ε**	**ENST00000115808**

La familia PPP2R2 (B/B55/PR55)

Los miembros de la familia PPP2R2 (B/B55/PR55/PPP2R2) están codificados por 4 genes distintos que dan lugar a 6 transcritos (Mayer et al., 1991), con una distribución intracelular distinta (Strack et al., 1998). Requieren de la metilación en L309 de la subunidad C para poder interaccionar con el núcleo enzimático A-C (Bryant et al., 1999). Presentan una expresión generalizada, aunque PPP2R2B y PPP2R2C se expresan más en cerebro (Strack et al., 1999; Zolnierowicz et al., 1994).

La familia PPP2R3 (B´´/PR72)

Esta familia incluye dos isoformas (PR72 y PR130) que están codificadas por el mismo gen aunque reguladas por promotores distintos, lo hace que difieran en su extremo amino-terminal. Además, presentan una distribución específica de tejido (Hendrix et al., 1993). Más tarde se describió un transcrito alternativo de PR70 llamado PR48. Todos los miembros comparten una estructura que contiene un dominio EFX, el cual en función de los niveles de Ca^{2+} regula la unión de estas isoformas B al heterodímero A-C (Janssens et al., 2003; Voorhoebe et al., 1999).

La familia PPP2R5 (B´/B56/PR61)

La familia PPP2R5 (B´/B56/PR61/PPP2R5) está formada por 5 genes diferentes con expresión diversa según tejido, que originan 8 transcritos que comparten una región central conservada y difieren en sus extremos amino y carboxilo. Se diferencian mucho de las otras familias de subunidades B. En su estructura incluyen un surco acídico por el que interaccionan con los correspondientes substratos, y su distribución intracelular es diferente. Así, mientras PPP2R5A, PPP2R5B y PPP2R5E se expresan en el citoplasma, PPP2R5C lo hace en el núcleo, y PPP2R5D está presente en ambos. Se ha visto que el tratamiento con ácido retinoico induce la expresión de PPP2R5B y PPP2R5D, lo que sugiere un papel de estas isoformas en el desarrollo (McCright et al., 1995; McCright et al., 1996; Csortos et al., 1996).

PTPA/PR53/PPP2R4

La proteína PTPA ha sido incluida en este apartado porque inicialmente fue descrita como una subunidad B por su capacidad de interacción con la subunidad A estructural. Se ha descrito que PTPA (Protein phosphatase 2A activator) regula la actividad de PP2A y es capaz de inducir en PP2A una actividad fosfotirosil-fosfatasa. Existen al menos 4 isoformas distintas fruto de procesos de transcripción alternativa y su expresión es generalizada (Goris et al., 1988; Cayla et al., 1990; Janssens et al., 2000).

1.4 Heterogeneidad de los complejos PP2A

Si a este número de isoformas diferentes de cada una de las subunidades de PP2A le sumamos la variabilidad aportada por los procesos de corte y empalme alternativo de exones (*splicing*), es fácil intuir la enorme cantidad de complejos heterotriméricos diferentes que pueden formarse por la combinación de isoformas concretas de cada una de las subunidades, lo que indica la gran complejidad del

estudio de PP2A. En concreto, si asumimos que no existen diferencias significativas entre Cα y Cβ, serían posibles 30 combinaciones distintas de complejos PP2A, y eso sin contar los procesos de *splicing* alternativo. A nivel funcional, si tenemos en cuenta la posible redundancia funcional entre algunos miembros de una misma familia y la expresión específica de tejido, probablemente el número de complejos PP2A diferentes se reduzca, aunque tampoco se sabe con certeza si ya han sido identificadas todas las subunidades existentes. De este modo, con el paso del tiempo, se han ido identificando complejos PP2A concretos implicados en la regulación de muy diversas funciones celulares y rutas de señalización, así como la desregulación de los mismos asociada a distintos aspectos del proceso de transformación celular.

2. Papel de PP2A en cáncer

Los procesos reversibles de fosforilación/desfosforilación constituyen la primera modificación post-traduccional descrita capaz de alterar el estado de activación de una enzima (Nolan et al., 1964). La desregulación de este proceso por alteraciones que afectan a quinasas y fosfatasas es algo característico de la célula tumoral, lo cual convierte a algunas de estas moléculas en dianas terapéuticas para terapias antitumorales de muchos tipos de cánceres (Dancey et al., 2003; Sebolt-Leopold et al., 2004). Aunque el papel de las quinasas en cáncer ha sido ampliamente estudiado, todavía existe un gran desconocimiento sobre la importancia de las fosfatasas en el desarrollo tumoral. Durante el proceso de transformación maligna tiene lugar en las células una acumulación de alteraciones genéticas y epigenéticas, y cada vez hay más consenso acerca de la existencia de un conjunto de alteraciones necesarias para la transformación celular, tales como la inactivación de Rb y p53, la activación de Ras o la expresión de la enzima telomerasa (Hanahan et al., 2000; Hahn et al., 2002). Estos cambios resultan suficientes para inmortalizar las células; sin embargo, no provocan una transformación completa (definida como capacidad de crecimiento independiente de anclaje y formación de tumores en ratones inmunodeficientes) (Sablina et al., 2007). La serina-treonina fosfatasa 2A (PP2A) está implicada en la regulación de una amplia variedad de procesos celulares y vías de señalización, cuya desregulación contribuye al proceso de transformación celular. De hecho, cada vez más estudios apuntan al papel fundamental de PP2A como supresor tumoral y a la inhibición de la actividad de PP2A como una alteración adicional necesaria para la progresión a célula maligna.

Los primeros indicios que apuntaban a PP2A como supresor tumoral vinieron de estudios con el ácido okadaico, un inhibidor de PP2A con capacidad de promover el crecimiento tumoral en ratones (Suganuma et al., 1988; Bialojan et al., 1988; Suganuma et al., 1990; Fujiki et al., 1993), y del antígeno tumoral pequeño (ST) del virus SV40, implicado en la trasformación de células humanas. ST-SV40 inhibe la actividad de PP2A al unirse a la subunidad A estructural y desplazar la subunidad B reguladora del complejo heterotrimérico PP2A y, de este modo, aumenta la fosforilación de proteínas implicadas en proliferación como AKT (Pallas et al., 1990; Rodriguez-Viciana et al., 2006; Andrabi et al., 2007). Así, las propiedades oncogénicas del ácido okadaico y de ST-SV40 indican que la transformación celular vía PP2A también podría tener lugar a través de otros mecanismos que inhiban la actividad de PP2A.

2.1. Actividad supresora tumoral de las subunidades de PP2A

Son muchos los trabajos que a lo largo del tiempo han ido implicando a complejos PP2A concretos en la regulación de rutas de señalización claves para la transformación celular y que señalan a PP2A en conjunto y, en particular a muchas de sus subunidades, como supresores tumorales. Entre los mecanismos que las células cancerosas emplean para inhibir la actividad de PP2A se encuentran la reducción de la expresión de determinadas subunidades de PP2A, la presencia de alteraciones genéticas afectando a dichas subunidades, y la activación de inhibidores endógenos de PP2A. Además, se ha descrito que la expresión ectópica de subunidades B reguladoras hace que éstas compitan en su unión con el núcleo enzimático A-C (Yang et al., 2003), por lo que la presencia de isoformas B alteradas podrían no sólo bloquear la función llevada a cabo por los complejos PP2A que las incluyan, sino también reducir la capacidad de otras isoformas B diferentes para reclutar al heterodímero A-C y por tanto, su actividad como supresores tumorales.

En relación con su papel en cáncer, se ha demostrado la importancia de PP2A en la regulación de muchas vías de señalización, entre las que destacan las rutas MAPK y WNT (Figuras 3 y 4). PP2A puede regular positiva o negativamente la vía RAF-MEK-ERK, y la regulación en uno u otro sentido podría estar determinada por el tipo celular, el tipo de estímulo, y la composición del complejo PP2A, sobre todo en lo que respecta a la subunidad B reguladora (Figura 3).

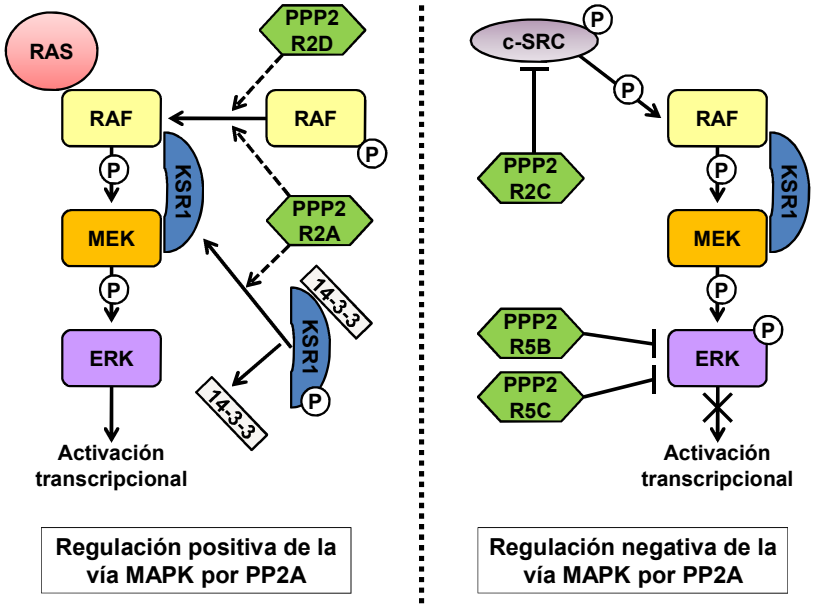


Figura 3. PP2A regula tanto positiva como negativa sobre la vía de señalización MAPK. Tras la estimulación, RAS promueve la unión de PPP2R2A tanto a RAF como a KSR1, lo cual lleva a su desfosforilación en las S392 y 295, respectivamente. Esto provoca que RAF transloque a membrana y se produzca la interación RAS-RAF. Además, la pérdida de la inhibición de la proteína 14-3-3 sobre KSR1 permite que KSR1 ejerza su función estructural y se active la cascada MAPK. Además, la proteína RAF puede también ser desfosforilada en S295 por PPP2R2D. Por el contrario, PP2A puede regular negativamente la vía MAPK a través de la desfosforilación y consiguiente inhibición de la quinasa c-SRC, la cual es capaz de activar a RAF de modo independiente de RAS, activando en último término a ERK. Además, ERK puede ser inhibido directamente por la acción de las subunidades PP2A reguladoras PPP2R5B y PPP2R5C (adaptada de Eichhorn et al., 2009).

La vía de señalización WNT/Beta-catenina es fundamental durante las primeras fases del desarrollo, y su desregulación en células de tejido adulto puede llevar a una proliferación incontrolada, una característica de la célula tumoral (Barker et al., 2000). Al igual que ocurre con la vía MAPK, en la ruta WNT el efecto regulador de PP2A puede ser tanto activador como represor. Sin embargo, la inhibición de PP2A tras el tratamiento con ácido okadaico lleva a una estabilización y acumulación de beta-catenina, lo que parece indicar que el efecto dominante de PP2A sobre esta ruta es el inhibidor (Seeling et al., 1999) (Figura 4).

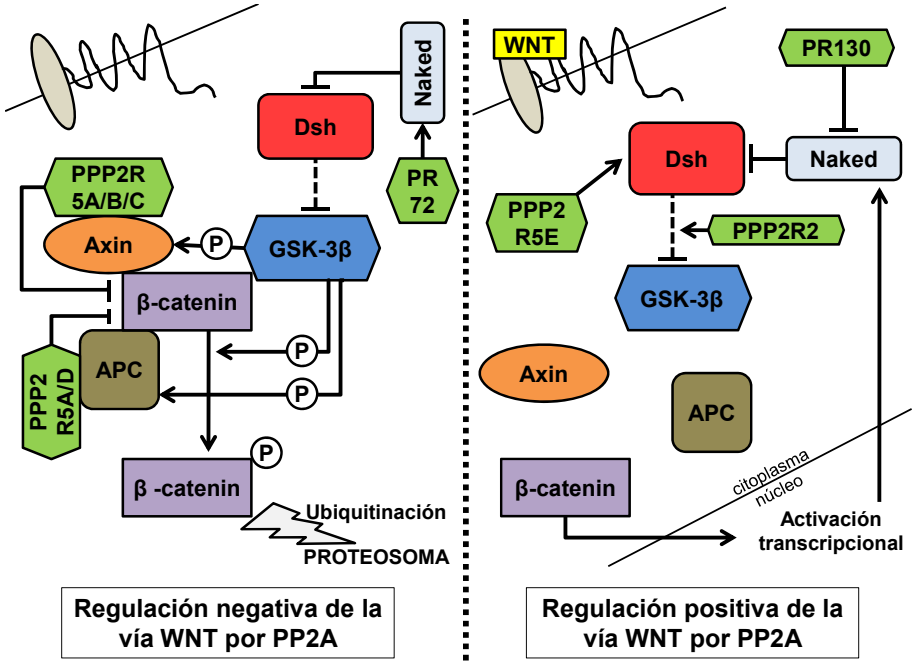


Figura 4. PP2A puede ejercer una regulación tanto positiva como negativa sobre la vía de señalización WNT. En ausencia de señal WNT, la proteína β-catenina forma un complejo con APC, Axin y GSK-3β en el citoplasma. GSK-3β regula negativamente por fosforilación a β-catenina, marcándola para su posterior ubiquitinación y degradación en el proteosoma. Las subunidades de PP2A reguladoras PPP2R5A y PPP2R5D se unen a APC e inducen una disminución de los niveles de β-catenina. Un efecto similar es producido tras la unión a Axin de las subunidades PPP2R5A, PPP2R5B y PPP2R5C. El mecanismo molecular exacto por el cual estas subunidades reguladoras de PP2A inducen la reducción de niveles de β-catenina permanece desconocido. Tras la señal WNT, el complejo APC-Axin-GSK-3β es degradado por Dsh, y la β-catenina desfosforilada transloca al núcleo, produciéndose la activación transcripcional de genes diana de la ruta WNT. Naked, uno de los genes diana de la ruta WNT y que ejerce un efecto inhibidor sobre Dsh, es activado por la subunidad de PP2A reguladora PR72 e inhibido por PR130. Por otra parte, la subunidad reguladora PPP2R5E regula positivamente la actividad de Dsh, inhibiendo de este modo la acción de GSK-3β, al igual que las subunidadesdel tipo PPP2R2, y de este modo regula positivamente la ruta WNT (adaptada de Eichhorn et al., 2009).

En un intento por recopilar los datos aportados por los numerosos estudios sobre PP2A que se han llevado a cabo, se expone a continuación la implicación a nivel individual de las distintas subunidades de PP2A en cáncer.

A. Subunidades estructurales

A pesar de que Aα y Aβ comparten un 87% en identidad de secuencia (Hendrix et al., 1993), los complejos PP2A a los que dan lugar están implicados en la

regulación de vías de señalización diferentes dentro de la célula (Figura 5). Así, mientras Aα parece centrar su acción en la ruta de AKT y otras proteínas como APC y p53, en la transformación mediada por la inactivación de Aβ parece ser RalA la que juega un papel fundamental. Distintas observaciones parecen confirmar que Aα y Aβ funcionan a través de mecanismos diferentes, ya que la pérdida de Aβ tiene un potencial transformante que no se bloquea con la expresión ectópica de Aα. Además, se ha visto que ST interacciona con Aα pero no con Aβ, y que RalA lo hace con Aβ pero no con Aα (Sablina et al., 2007).

Isoforma Aα

Se ha descrito la presencia de mutaciones en la isoforma Aα de la subunidad A estructural en varios tipos de cáncer, como melanoma, cáncer de pulmón y cáncer de mama. Estas mutaciones afectan a la capacidad de formar complejos heterotriméricos PP2A, ya que impiden su unión a las subunidades C catalítica y B reguladora (Calin et al., 2000; Ruediger et al., 2001) y por lo tanto, se disminuye la actividad de PP2A y se activa la ruta AKT. La muerte celular por apoptosis tras la pérdida completa de Aα indica que se trata de un gen esencial para la célula y que al menos se requieren unos niveles basales de expresión del mismo (Strack et al., 2004; Arroyo et al., 2005). Sin embargo, la reducción de la expresión de Aα en un 50% es capaz de sustituir a ST-SV40 en la transformación de células HEK TER (que expresan LT, hTERT y H-Ras) (Chen et al., 2005), lo que sugiere que las mutaciones en Aα estarían contribuyendo a la transformación celular mediante la creación de un estado de haploinsuficiencia, que estaría desregulando la ruta PI3K (Sablina et al., 2007). Además, esta supresión del 50% en la expresión de Aα lleva a la ausencia de complejos PP2A del tipo Aα-C-PPP2R5C, de un modo similar a la supresión de PPP2R5C (Chen et al., 2005). Esto sugiere que este tipo de complejos PP2A estarían implicados en la regulación por fosforilación de sustratos importantes en el proceso de transformación celular entre los que se encuentra AKT, APC y p53.

Isoforma Aβ

Además de las alteraciones descritas para Aα, se han descrito también mutaciones que afectan a la subunidad estructural Aβ en cáncer de mama, pulmón, colon, leucemia linfática crónica de células B, carcinoma hepatocelular, hiperplasia paratiroidea y adenoma paratiroideo (Calin et al., 2000; Takagi et al., 2000; Wang et al., 1998; Hemmer et al., 2002; Kalla et al., 2007; Chou et al., 2007). Estas mutaciones afectan a la capacidad de interacción de Aβ con las subunidad C catalítica y B reguladora (Ruediger et al., 2001; Esplin et al., 2006). Sin embargo, a diferencia del estado de haploinsuficiencia creado por las mutaciones de Aα, en el caso de la isoforma Aβ, las mutaciones se acompañan a menudo de la pérdida del segundo alelo, por lo que en los casos en los que el alelo mutado no sea funcional se podría hablar de una inactivación completa de Aβ (Calin et al., 2000; Takagi et al., 2000; Wang et al., 1998). Se ha descrito que la expresión ectópica de Aβ en células de cáncer de pulmón portadoras de mutaciones bialélicas de Aβ revierte parcialmente su fenotipo tumoral, y que la supresión de Aβ coopera con el antígeno tumoral grande (LT), hTERT and H-Ras en la transformación de células HEK TER (Sablina et al., 2007).

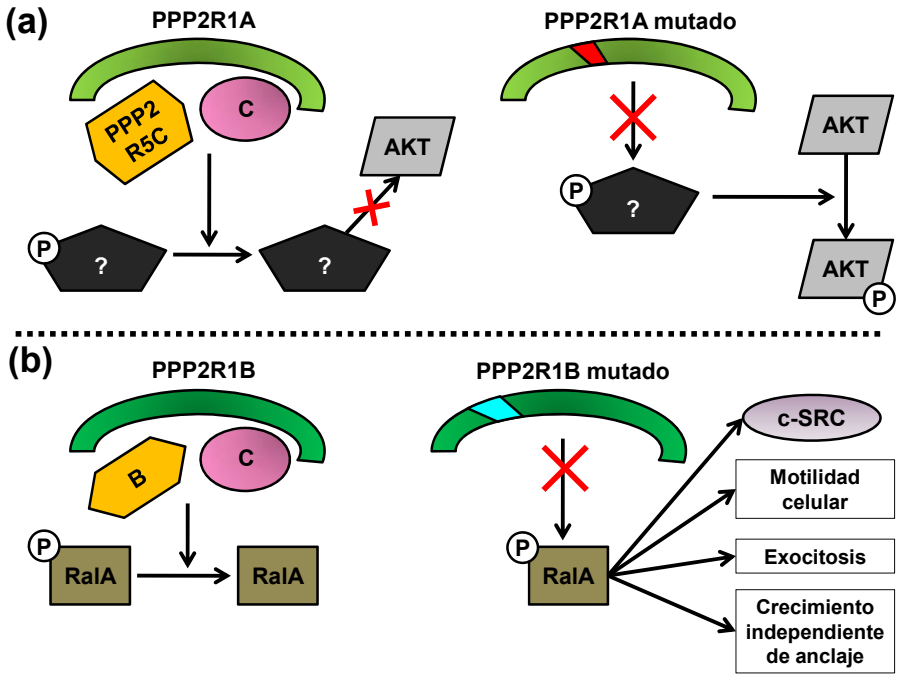


Figura 5. Las subunidades A estructurales actúan como supresores tumorales por mecanismos distintos e independientes; (a) Aα regula la fosforilación de AKT a través de un intermediario todavía desconocido. Las mutaciones en Aα crean un estado de haploinsuficiencia con pérdida de complejos PP2Ac/Aα/PPP2R5 y aumento de fosforilación de AKT; (b) Aβ se une y desfosforila a la GTPasa RalA. Las alteraciones en Aβ suelen afectar a ambos alelos y provocan un incremento de los niveles de fosforilación de RalA (adaptada de Westermarck y Hahn, 2008).

Por otra parte, Aβ inhibe a la GTPasa RalA por desfosforilación en las serinas 183 y 194. RalA está implicada en la activación de la quinasa c-SRC (Goi et al., 2000), motilidad celular (Ohta et al., 1999; Gildea et al., 2002), exocitosis (Moskalenko et al., 2002) y crecimiento independiente de anclaje (Chien et al., 2003; Lim et al., 2005. Además, se ha descrito la relación de RalA con la adquisición del fenotipo de célula transformada en varias líneas celulares y con la activación de la vía Ras (Chien et al., 2003; Lim et al., 2005; Hamad et al., 2002).

B. Subunidades reguladoras

Familia PPP2R2 (B/B55/PR55)

Ya hemos visto como la familia de subunidades reguladoras PPP2R2 ejerce una regulación de las vías de señalización MAPK y WNT (Figuras 3 y 4).

La subunidad reguladora PPP2R2A es reclutada a la membrana plasmática en respuesta a factores de crecimiento como PDGF, hacia complejos que contienen el núcleo enzimático A-C de PP2A, y las proteínas KSR1 (Kinase Suppressor of Ras) y RAF-1 (Ory et al., 2003; Adams et al., 2005), produciéndose la desfosforilación de las mismas, con la consecuente activación de la vía MAPK. Sin embargo, tanto la inhibición de PPP2R2 por ST (Sontag et al., 1993) como por el tratamiento con ácido okadaico (Sonoda et al., 1997) provocan la fosforilación y activación de la quinasa ERK, la cual se sitúa en señalización cascada abajo de RAF-1. Además, en consonancia con estos datos, tanto la supresión de la subunidad C catalítica, la subunidad A estructural o la familia de subunidades B reguladoras PPP2R2 provoca la activación de la vía MAPK en

respuesta a insulina en células de Drosophila (Silverstein et al., 2002). Por lo tanto, parece que PP2A podría ejercer tanto un efecto activador como inhibidor sobre la vía MAPK (Wassarman et al., 1996). Otra función de PPP2R2A está en relación con la apoptosis inducida por la proteína adenoviral E4orf4 (Shtrichman et al., 2000). Se ha comprobado que PPP2R2D, al igual que PPP2R2A, regula positivamente la ruta MAPK a través de la desfosforilación de RAF-1 en S259 (Adams et al., 2005).

Por lo que respecta a la isoforma PPP2R2C, se ha descrito que interacciona de forma directa con c-SRC regulando negativamente su actividad (Eichhorn et al., 2007). Por su parte, c-SRC podría activar a RAF-1 independientemente de RAS (Chao et al., 1997; Stokoe et al., 1997), y de ese modo activar la vía MAPK (Ziogas et al., 2005). La supresión de PPP2R2C ó PPPP2R2D en situación de estrés celular lleva a un incremento de la activación de la ruta de estrés JNK dependiente de SRC que conduce a apoptosis (Eichhorn et al., 2007).

Familia PPP2R3 (B´´/PR72)

La proteína Naked es un regulador negativo de la vía WNT (Zeng et al., 2000) que requiere para su función de la presencia de la subunidad reguladora PR72 de la familia PPP2R3 (Creyghton et al., 2005). Tanto Naked como PR72 incluyen en su estructura un dominio EFX, lo que sugiere que están reguladas por los niveles de calcio intracelulares (Ikura, 1996) (Figura 4). La isoforma PR130, codificada por un transcrito alternativo más largo de PR72, interacciona con Naked y la inhibe, ejerciendo un efecto activador de la vía WNT (Creyghton et al., 2006). Además, la supresión mediante shRNAs de los complejos PP2A con subunidades reguladoras PR72 y PR130 ha demostrado que están implicados en transformación celular y en la regulación de las vías de señalización de c-MYC y WNT (Sablina et al., 2010).

Familia PTPA/PR53/PPP2R4

La inhibición de la expresión de PTPA ha permitido identificar a esta proteína como implicada en la transformación celular y en la regulación de las vías de señalización de c-MYC y de WNT, y también de PI3K mediante la desfosforilación de AKT (Sablina et al., 2010).

Familia PPP2R5 (B´/B56/PR61)

La expresión ectópica de los miembros de la familia PPP2R5 inhibe la señalización WNT mediante la disminución de los niveles de beta-catenina (Seeling et al., 1999). Además, mediante experimentos de supresión con shRNAs se ha identificado que los complejos PP2A con subunidades reguladoras PPP2R5A y PPP2R5C están implicados en la transformación celular y en la regulación de las vías de señalización de c-MYC, WNT y PI3K (Sablina et al., 2010).

PPP2R5A

Los complejos PP2A/PPP2R5A participan en la regulación de c-MYC, BCL-2, p53, AKT y posiblemente BAD. c-MYC está sobreexpresado en muchos tipos de cáncer (Adhikary et al., 2005). Su vida media en células no transformadas es muy corta pero está incrementada en líneas celulares neoplásicas (Gregory et al., 2000; Malempati et al., 2006). Su estabilidad está regulada por los niveles de fosforilación en T58 y S62. La fosforilación en T58 por GSK-3β marca a c-MYC

para la degradación, mientras que la activación de la ruta ERK/MAPK induce la fosforilación en S62, aumentando la estabilidad de c-MYC (Sears et al., 1999; Sears et al., 2000). La proteína c-MYC es diana directa de los complejos PP2A que tienen una subunidad reguladora de tipo PPP2R5A (PP2A/PPP2R5A). Estos complejos PP2A/PPP2R5A desfosforilan a c-MYC en S62, marcándolo de este modo para su ubiquitinación y posterior degradación en el proteosoma. Por tanto, la inhibición de PPP2R5A se traduce en un aumento tanto de los niveles de c-MYC como de su actividad (Arnold et al., 2006).

Otra diana conocida de PPP2R5A es la proteína anti-apoptótica BCL-2, a la que desfosforila e inactiva (Ruvolo et al., 2002). Por otra parte, se ha descrito que los complejos PP2A/PPP2R5A regulan negativamente a p53 vía MDM2 (Haupt et al., 1997), en contraste con la regulación positiva descrita en el caso de los complejos PP2A/PPP2R5C. PP2A/PPP2R5A también desfosforila en T308 e inhibe al oncogén AKT, desregulado con frecuencia en muchos tipos de cáncer por su papel activador de vías de señalización implicadas en la proliferación celular (Bellacosa et al., 1995; Kuo et al., 2007). Además, PP2A activa a la proteína pro-apoptótica BAD y aunque el complejo específico PP2A que realiza esta función aún no ha sido determinado, un candidato es PP2A/PPP2R5A (Chiang et al., 2003).

PPP2R5B

También parece que PP2A regula a ERK, ya que la supresión de PPP2R5B o de PPP2R5C lleva a un incremento en la actividad de ERK pero no de MEK, que es la quinasa que está en la señalización justo por encima de ERK, lo que indicaría una acción directa de PP2A sobre ERK (Letourneux et al., 2006).

PPP2R5C

Entre los indicios que señalan a PPP2R5C como supresor tumoral está que su supresión, del mismo modo que ST-SV40, induce transformación en células HEK TER (Chen et al., 2004). Además, la expresión de PPP2R5C es baja en melanomas (Deichmann et al., 2001), y se ha comprobado la capacidad metastática adquirida por un subclón de melanoma de ratón B16 con una forma truncada de PPP2R5C. En este trabajo, los autores vieron que la forma truncada de PPP2R5C interaccionaba en las adhesiones focales con la proteína Paxilina de modo similar a la forma no truncada, pero Paxilina se encontraba hiperfosforilada, lo que sugiere que en el complejo PP2A con la forma truncada de PPP2R5C se producía la unión con la proteína diana Paxilina pero la actividad del complejo estaba inhibida (Ito et al., 2000). Por otra parte, en muchas líneas celulares de cáncer de pulmón en las que está suprimida la expresión proteica de PPP2R5C, su expresión ectópica es capaz de revertir parcialmente el fenotipo tumoral de dichas células (Chen et al., 2004).

También, se ha comprobado que los complejos PP2A con una subunidad reguladora de tipo PPP2R5C inhiben la formación de complejos APC-axin, llevando a la desestabilización de la beta-catenina, lo cual reduce su abundancia y los niveles de transcripción de sus genes diana (Polakis, 2000) (Figura 4). Otra función de los complejos PP2A/PPP2R5C es participar en la regulación de p53, lo que llevan a cabo mediante 2 mecanismos distintos. Por una parte, PP2A/PPP2R5C desfosforila a p53 en T55, previniendo de este modo su degradación por el proteosoma e inhibiendo además la proliferación al inducir la expresión de p21 (Li et al., 2007). Por otra parte, el reclutamiento de

PP2A/PPP2R5C por Ciclina-G hacia complejos que contienen p53 y MDM2, lleva a la desfosforilación de Mdm2, lo cual estabiliza a p53 (Okamoto et al., 2002). En relación con el control del ciclo celular, recientemente se ha demostrado que PPP2R5C regula la acumulación de PP2A en el núcleo, así como la transición de G1 a S (Lee et al., 2010).

PPP2R5D

Se ha visto que PPP2R5D interacciona con la proteína APC y participa en la regulación negativa de la vía de señalización WNT (Seeling et al., 1999).

PPP2R5E

En un principio, PPP2R5E fue descrito como un inhibidor de la vía WNT (Seeling et al., 1999); sin embargo, experimentos posteriores han mostrado que PPP2R5E regula positivamente la señalización WNT durante el desarrollo en vertebrados (Yang et al., 2003). Se ha propuesto que estas contradicciones podrían deberse a que cuando una subunidad reguladora se sobreexpresa ejerce un efecto dominante negativo al secuestrar proteínas diana o componentes del complejo PP2A, o simplemente al desplazar a otras subunidades B reguladoras, por lo que habría que confirmar los resultados obtenidos con experimentos de inhibición (Yang et al., 2003). Recientemente, se ha publicado que PPP2R5E puede llevar a cabo funciones pro- y anti-apoptóticas, lo que indica su posible implicación en procesos de desarrollo tumoral (Jin et al., 2010). Además, se ha comprobado que el miR-19b regula a PPP2R5E, y parece que los efectos oncogénicos de este microRNA podrían venir en parte por la inhibición de PPP2R5E (Mavrakis et al., 2010). Esta es la primera evidencia de una regulación de PP2A por microRNAs, y añade un nivel aún mayor de complejidad a la regulación de la actividad PP2A.

2.2. Proteínas inhibidoras de PP2A

Además de las alteraciones en las distintas subunidades que forman el complejo PP2A, otro mecanismo que tiene la célula para inhibir la actividad supresora tumoral de PP2A es la desregulación de proteínas inhibidoras de PP2A. A continuación, se exponen los principales inhibidores endógenos de PP2A, así como su implicación en cáncer.

CIP2A

La proteína CIP2A (*Cancerous Inhibitor of PP2A*) fue identificada como una proteína que interacciona con PP2A dando lugar a la estabilización de c-MYC en células transformadas, ya que inhibe la desfosforilación de c-MYC en S62 por PPP2R5A (Junttila et al., 2007) (Figura 6). Teniendo en cuenta que la supresión de CIP2A no tiene ningún efecto en la actividad de PP2A sobre MDM2, parece que la capacidad de CIP2A de inhibir a PP2A es dependiente de su interacción con MYC, por lo que sólo tendría efecto sobre la actividad de PP2A sobre MYC (Junttila et al., 2007).

La expresión de *CIP2A* en tejidos normales es muy baja; sin embargo, hay sobreexpresión de *CIP2A* en algunos tipos de cáncer (Soo Hoo et al., 2002; Junttila et al., 2007; Li et al., 2008; Vaarala et al., 2010; Qu et al., 2010; Wang et al., 2011), y se asocia con mal pronóstico en pacientes con carcinoma gástrico y

cáncer de pulmón de células no pequeñas (Khanna et al.,, 2009; Dong et al., 2010), y con mayor agresividad en cáncer de mama (Come et al., 2009).

Por último, la expresión ectópica de *CIP2A* aumenta la capacidad proliferativa y de autorenovación de progenitores neuronales de ratón (Kerosuo et al., 2010). Además, *CIP2A* se ha identificado en una traslocación con MLL en un caso de leucemia mieloide aguda infantil (Coenen et al., 2011), y se ha comprobado que media en los efectos del bortezomib en cáncer hepatocelular (Chen et al., 2010).

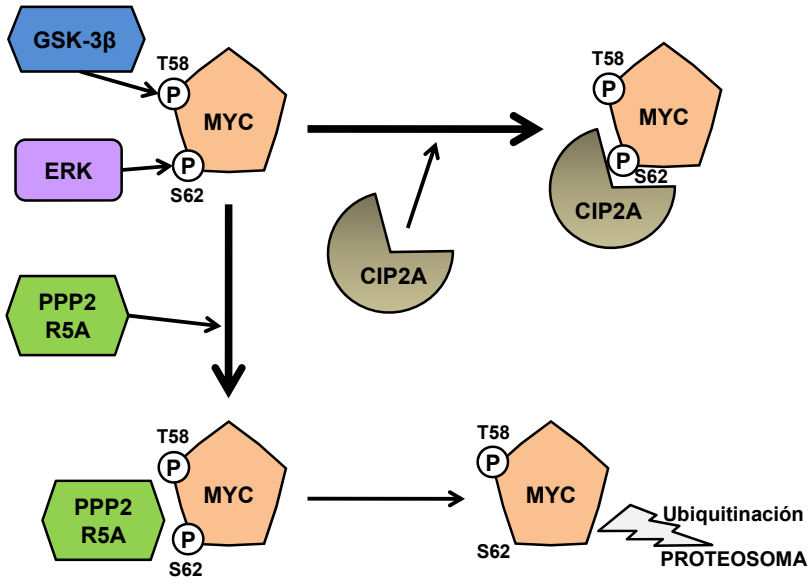


Figura 6. Papel de CIP2A en la regulación de los niveles de MYC. GSK-3β y ERK fosforilan a MYC en T58 y S62 respectivamente. El complejo PP2A se une a MYC por medio de PPP2R5A y lo desfosforila en S62, lo cual marca a MYC para su ubiquitinación y posterior degradación en el proteosoma. Sin embargo, en presencia de CIP2A se inhibe la acción de PP2A sobre MYC, lo cual lleva a su estabilización (adaptada de Westermarck y Hahn, 2008).

SET (I2PP2A/TAF-1β)

Las proteínas I1PP2A y SET (I2PP2A/TAF-1β) funcionan como inhibidores endógenos no competitivos de PP2A (Li et al., 1995; Li et al., 1996). Además, se ha comprobado que I1PP2A y SET también se asocian con PP1 y, de hecho, en presencia de Mn^{2+}, activan selectivamente a PP1 (Katayose et al., 2000).

La proteína SET participa en la regulación de una gran cantidad de funciones dentro de la célula, tales como replicación del DNA, remodelación de la cromatina y transcripción génica (Seo et al., 2001; Kutney et al., 2004), diferenciación (Kandilci et al., 2004), y regulación del ciclo celular (Canela et al., 2003). Además, inhibe la actividad DNasa del supresor tumoral NM23-H1 (Fan et al., 2003).

El gen que codifica SET fue implicado por primera vez en cáncer al describirse fusionado a la nucleoporina Nup214 en leucemia mieloide aguda (von-Lindern et al., 1992), y está sobreexpresado en varios tipos de cáncer (Cervoni et al., 2002; Ginos et al., 2004; Korkola et al., 2006; Andersson et al., 2007), lo que llevaría a una inactivación de PP2A.

La vía MAPK es una de las principales dianas de la actividad PP2A, cuya inhibición por ácido okadaico da lugar a una activación de AP-1 (Westermarck et al., 1998; Junttila et al., 2008). De acuerdo con esto, la sobreexpresión de SET induce la fosforilación de c-JUN en S63 y T73, y activa AP-1 (Al-Murrani et al., 1999) (Figura 7). Se ha comprobado que la expresión de SET está muy reducida en células quiescentes debido a ausencia de suero, inhibición por contacto o diferenciación, lo cual sugiere un papel de SET como regulador positivo de la proliferación celular (Carlson et al., 1998).

En leucemia mieloide crónica (LMC), BCR-ABL inhibe a PP2A a través de la sobreexpresión de SET. La inducción de SET por BCR-ABL aumenta la estabilidad del propio BCR-ABL, mientras que el silenciamiento de SET provoca su degradación. Además, la activación de PP2A por el silenciamiento de SET lleva a una menor expresión de MYC y a una desfosforilación de STAT5, ERK, AKT, BAD, JAK2 y RB, así como del propio BCR-ABL, aunque este último efecto parece estar mediado por la fosfatasa SHP-1 (Neviani et al., 2005).

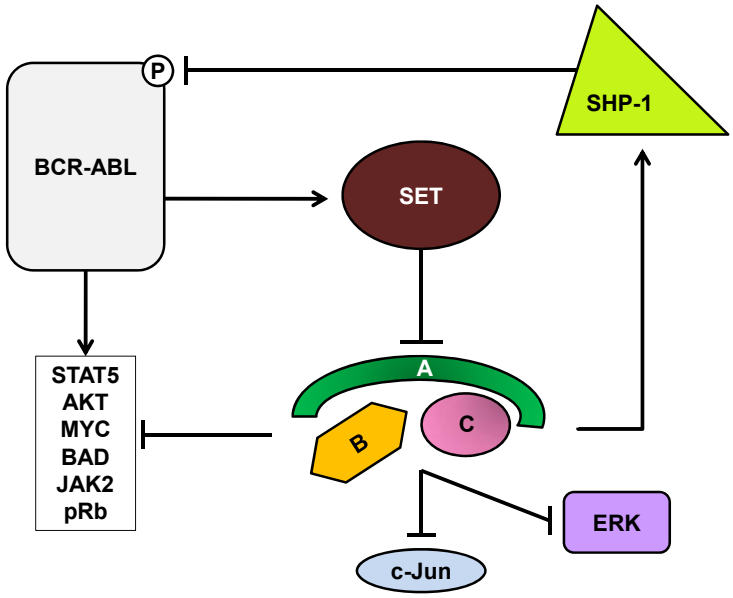


Figura 7. Rutas de señalización en las que está implicado SET como inhibidor de PP2A. BCR-ABL inhibe la actividad PP2A a través de la inducción de SET, protegiendo de la desfosforilación mediada por PP2A a algunas de sus dianas moleculares como STAT5, AKT, BAD, JAK2, pRB y también a sí mismo, aunque en este último caso el efecto parece estar mediado por la acción de SHP-1. Además, la sobreexpresión de SET protege también de su desfosforilación e inhibición a c-Jun y a ERK (adaptada de Westermarck y Hanh, 2008).

Otros inhibidores de PP2A

Se han descrito otros inhibidores de PP2A como JAK2, PME-1 y TIP. Se ha comprobado que la quinasa JAK2 fosforila directamente a PP2A en la Y307 de su subunidad C catalítica, inhibiendo su actividad (Yokoyama et al., 2003). La metilesterasa PME-1 se une con la subunidad C catalítica de PP2A y la inhibe por la desmetilación en su L307 (Longin et al., 2004). La proteína TIP interacciona con PP2A e inhibe su acción en la vía ATM/ATR (McConnell et al., 2007). Además, como se ha indicado anteriormente, entre los inhibidores de PP2A está la

oncoproteína ST y el agente químico ácido okadaico (Katoh et al., 1990; Sontag et al., 1993).

2.3. Papel de PP2A en leucemia

Como se acaba de señalar, en la LMC, la proteína de fusión BCR-ABL inhibe PP2A a través de un aumento de los niveles de expresión de SET. De hecho, los niveles de SET se correlacionan con la progresión de la enfermedad, de modo que se produce un incremento considerable en el paso de fase crónica a crisis blástica, como consecuencia de una mayor actividad BCR-ABL.

Además, la activación farmacológica de PP2A mediante forskolina o la supresión de su inhibidor SET en células BCR-ABL positivas provoca un bloqueo de la proliferación celular y un aumento de la diferenciación y apoptosis, que tiene como consecuencia una disminución de la capacidad de estas células para desarrollar leucemia en modelos *in vivo*. Otra consecuencia es la desfosforilación de proteínas diana de PP2A importantes para el desarrollo de la leucemia, como ERK, AKT, MYC, BAD, JAK2 y RB (Neviani et al., 2005). Por otra parte, el activador de PP2A FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) se ha mostrado efectivo tanto en células de pacientes con CML y ALL-Ph+, como en modelos *in vivo* e *in vitro* de ambas leucemias, reduciendo el potencial leucémico de dichas células e induciendo su entrada en apoptosis, efecto que no ejerce sobre células normales CD34+/CD19+ de médula ósea (Neviani et al., 2007). Teniendo en cuenta estos resultados, el tratamiento con activadores de PP2A podría ser una alternativa terapéutica en pacientes con este tipo de leucemias (Figura 8).

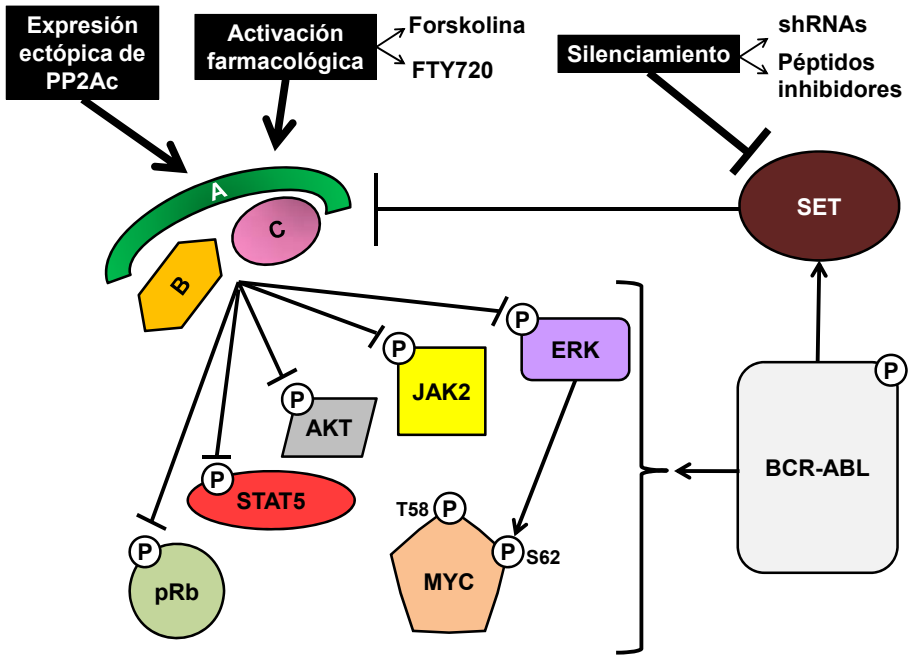


Figura 8. Importancia del estado de activación de PP2A en células BCR-ABL positivas de LMC y LLA (adaptada de Perrotti y Neviani, 2008).

Hay sólo dos estudios sobre el papel de PP2A en LMA. Un trabajo en una serie pequeña de pacientes con LMA comprobó que los niveles de fosforilación en

T308 de AKT se asociaban con peor pronóstico y cariotipo complejo, y encontraron que la fosforilación en T308 se correlacionaba con la actividad PP2A (Gallay et al., 2009). Además, un estudio reciente ha mostrado que las mutaciones activadoras de c-KIT llevan a la inhibición de PP2A, y que su reactivación es capaz de suprimir el crecimiento de células c-KIT+ tanto in vivo como in vitro, lo que indica que la inhibición de PP2A podría ser un paso clave en el desarrollo de leucemias c-KIT+ (Roberts et al., 2010).

Estos estudios podrían tener importancia en el futuro tratamiento de estos pacientes ya que hay compuestos, como forskolin y FTY70, que son activadores de PP2A. El FTY720 se desarrolló originalmente como agente inmunosupresor, pero se vio que inducía respuesta en varios tipos de cáncer (Neviani et al., 2007; Sonoda et al., 2001; Azuma et al., 2002; Lee et al., 2005; Yasui et al., 2005). Se ha comprobado el potencial terapéutico de estos activadores de PP2A en LMC, LAL-Ph+, y en leucemia linfocítica crónica de células B (Neviani et al., 20005; Neviani et al., 2007; Liu et al., 2008).

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS

La leucemia mieloide aguda es una enfermedad heterogénea a nivel molecular, y distintas combinaciones de alteraciones genéticas y epigenéticas contribuyen al inicio y progresión de la enfermedad. En los últimos años, se han identificado varios marcadores genéticos con valor pronóstico en leucemia mieloide aguda y se han desarrollado nuevas terapias moleculares. Sin embargo, a pesar de los avances logrados, la supervivencia de los pacientes con esta enfermedad es muy baja. Por tanto, es fundamental la búsqueda de nuevas dianas y el desarrollo de nuevas estrategias de tratamiento que mejoren la supervivencia de estos pacientes.

Las tirosinas quinasas son proteínas que catalizan reacciones de fosforilación sobre otras proteínas. Su importancia en cáncer se debe a su papel regulador en procesos como proliferación, diferenciación o apoptosis. En leucemia mieloide aguda, las tirosinas quinasas han sido muy estudiadas. Algunas, como FLT3, juegan un papel importante en el desarrollo de la enfermedad y ya se están realizando ensayos clínicos para evaluar el potencial terapéutico de varios compuestos inhibidores de FLT3. Las fosfatasas catalizan la reacción contraria a la quinasas; sin embargo, los estudios sobre la implicación de estas proteínas en cáncer son mucho menos numerosos, especialmente en leucemia mieloide aguda. Entre ellas, destaca la proteína fosfatasa 2A (PP2A), que ejerce una función reguladora central en la célula, y cuya inactivación parece ser un evento esencial y un punto en común entre distintos tipos de cáncer. De hecho, se ha comprobado que su inhibición vía BCR-ABL juega un papel crucial en leucemia mieloide crónica y leucemia linfoblástica aguda Philadelphia positiva. Recientemente, también se ha descrito su inactivación en leucemia linfática crónica de células B, y en leucemias mieloides agudas con mutaciones de c-KIT. Además, su activación farmacológica en estos casos ha demostrado que PP2A podría ser una potencial diana terapéutica.

Dadas estas observaciones nos planteamos que la inactivación de PP2A podría ser también un evento importante en el desarrollo de la leucemia mieloide aguda. De este modo, nos propusimos abordar un estudio para determinar la importancia de la inhibición de PP2A en leucemia mieloide aguda, los mecanismos moleculares a través de los que tiene lugar y las posibilidades terapéuticas de la activación farmacológica de PP2A en esta enfermedad.

OBJETIVOS

1. Caracterizar genéticamente 12 líneas celulares de leucemia mieloide aguda y 4 de leucemia mieloide crónica en crisis blástica mediante la realización de arrays de expresión y de SNPs, y establecer los perfiles de expresión de 250 microRNAs mediante RT-PCR en tiempo real. Este estudio nos permitirá utilizar estas líneas mieloides en los siguientes estudios funcionales que nos proponemos realizar. Además, estudiaremos la desregulación de microRNAs debido a alteraciones genómicas por variaciones de número de copia, y llevaremos a cabo una correlación de los microRNAs afectados con la alteración de los niveles de expresión de sus genes diana. De este modo, esperamos identificar nuevos genes implicados en leucemia mieloide aguda cuya expresión esté desregulada por alteraciones en los microRNAs que controlan su expresión. Además del análisis bioinformático, se validarán los resultados obtenidos mediante análisis proteico y ensayos de luciferasa.

2. Estudiar la importancia de la inhibición de PP2A tanto en líneas celulares como en muestras de pacientes con leucemia mieloide aguda. Analizar el efecto de la activación farmacológica de PP2A mediante el tratamiento con los compuestos activadores de PP2A forskolina y FTY720, para determinar su valor potencial como alternativa terapéutica en leucemia mieloide aguda. Además, analizar el efecto de la combinación de forskolina y FTY720 con las drogas Idarrubicina y Ara-c, empleadas en el tratamiento estándar de inducción en pacientes con leucemia mieloide aguda.

3. Identificar los mecanismos de inhibición de PP2A en leucemia mieloide aguda y estudiar su recurrencia tanto en líneas celulares como en muestras de pacientes. Para ello, estudiaremos la presencia de alteraciones que afecten a las distintas subunidades de PP2A y los inhibidores endógenos de PP2A en la célula.

4. Estudiar el efecto de la expresión ectópica de SET, un potente inhibidor de PP2A, en líneas celulares y analizar si su desregulación es una alteración recurrente en leucemia mieloide aguda. Estudiar el valor pronóstico de dicha alteración y su asociación con marcadores moleculares con valor pronóstico en leucemia mieloide aguda. Determinar los mecanismos por los que SET está regulado a nivel transcripcional.

5. Se ha identificado en un paciente con leucemia mieloide aguda la sobreexpresión del gen SETBP1 (SET binding protein 1) como consecuencia de una traslocación t(12;18). Analizaremos el mecanismo molecular por el que SETBP1 lleva a la inhibición de PP2A y el papel que la interacción de SETBP1 con SET juega en el mismo. Además, estudiaremos la prevalencia de la sobreexpresión de SETBP1 y su valor como marcador pronóstico en una serie de pacientes con leucemia mieloide aguda al diagnóstico.

RESULTADOS:

ARTÍCULOS CIENTÍFICOS

<u>Capítulo 1.</u> Integration of global SNP-based mapping and expression arrays with microRNA patterns reveals deregulation of miR-370 and permits the identification of its target gene NF1 in acute myeloid leukemia

(artículo científico en preparación)

Integration of global SNP-based mapping and expression arrays with microRNA patterns reveals deregulation of miR-370 and permits the identification of its target gene *NF1* in acute myeloid leukemia

García-Ortí L*,[1] Cristóbal I*,[1,2] Cirauqui C, [1] Guruceaga E,[3] Marcotegui N,[1] Cortés X,[1,2] Alonso MM,[1] Odero MD[1,2]

[1]Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain

[2]Department of Genetics, University of Navarra, Pamplona, Spain

[3]Unit of Proteomics, Genomics and Bioinformatics, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain

*These authors have contributed equally to this work

Keywords: NF1, miR-370, AML

Running Title: NF1 regulation by miR-370 in AML

Scientific category: Myeloid Neoplasia

Corresponding author: María D. Odero, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pio XII-55 31008-Pamplona, Spain. E-mail: modero@unav.es. Phone: +34-948-194700. Fax: +34-948-194714.

Abstract

Deregulated miRNA expression has been largely reported to play a crucial role in tumorigenesis. Recent studies have shown different mechanisms leading to miRNA deregulation in cancer: mutations, chromosomal translocations, epigenetic alterations, and defective miRNA biogenesis. However, alterations affecting miRNAs by DNA copy number variations (CNV) remain poorly studied. Our aim was to identify if CNVs affect the expression levels of miRNAs in acute myeloid leukemia (AML), modulating the expression levels of their target genes. We analyzed 16 myeloid cell lines by an integrative approach including high resolution SNPs arrays, mRNA expression arrays, and quantification of 250 miRNAs by real-time PCR. We found correlation between the expression levels of 19 miRNAs and CNVs affecting the genomic regions in which these miRNAs are located. Furthermore, our integrative approach and functional analysis by western blot and luciferase assays confirmed that *NF1* is a direct target of miR-370, and that c-CBL is indirectly regulated by miR-379. Therefore, our approach showed that CNVs are alternative mechanisms that regulate miRNA expression levels in AML, and allowed the identification of novel candidate genes involved in AML.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disease characterized by enhanced proliferation and impaired differentiation of early progenitors. Its heterogeneity is caused by a variety of genetic and epigenetic aberrations that, acting in combination, contributes to the initiation and progression of this disease. In addition, it has been recently reported the implication of specific microRNAs (miRNAs) in the pathogenesis of AML (reviewed in Marcucci et al., 2011).

MiRNAs are small, non–coding RNAs that bind to the 3′-untranslated region of target genes negatively regulating their expression levels by either translation repression or mRNA degradation. MiRNAs are essential in key biological functions, such as cellular differentiation, development, stress response, apoptosis and cell growth (Garzon and Croce, 2008). In addition, miRNAs play important roles in normal hematopoiesis regulating hematopoietic differentiation, and their aberrant expression has been associated with hematological malignancies (Marcucci et al., 2011; Waldman and Terzic, 2009). For this reason, they are potential biomarkers and therapeutic agents in cancer. There are several mechanisms reported to lead to miRNA deregulation: mutations, chromosomal translocations, epigenetic alterations, or a defective miRNA biogenesis; however, little is known about the mechanisms of miRNA deregulation in AML (Garzon and Croce, 2008).

MiRNA microarrays in large series of AML patients have identified miRNA signatures associated with several cytogenetic and molecular groups (reviewed in Marcucci et al., 2011). Upregulation of a subset of miRNAs located in the human 14q32 imprinted domain, including miR-127, miR-154, miR-299, miR-323, miR-368 and miR-370 has been identified in cases with acute promyelocytic leukemia with t(15;17) (Dixon-McIver et al., 2008). In addition, overexpression of miR-126 in core-binding factor-AML (CBF-AML) is linked to RUNX1 fusion protein through targeting DROSHA (Yendamuri and Calin, 2009). Moreover, high expression of miR-199a and miR-191 was identified in AML patients with trisomy 8. One of the strongest miRNA signatures associates balanced 11q23 translocations with miR-196 downregulation (Garzon and Croce, 2008) and overexpression of the miR-17-92 polycistronic miRNA cluster (Li et al., 2008). Furthermore, AML patients carrying *NPM1* mutations had miR-204 downregulation and, as consequence, upregulation of their targets *HOXA10* and *MEIS1*, suggesting that the *HOX* upregulation seen in these patients may be secondary to miRNA alterations (Garzon et al., 2008). Recently, Becker et al. (2010) reported a miR-expression signature composed of 68 miRNAs (34 upregulated and 34 downregulated) associated with *NPM1* mutations in elderly patients with AML *de novo*. Importantly, *NPM1* mutation status could be predicted with accuracy based on miR-expression profiles (Becker et al., 2010). Apart from distinct miRNA signatures of interest for diagnosis and prognosis, the functional effects of some miRNA alterations have also been reported. For example, miR-155, which shows leukemogenic properties, has been found upregulated in AML patients with high white count and *FLT3*-ITD (O´Connell., 2008). *CEBPA* mutations have been associated with the upregulation of miR-181a and miR-335, and consequently, implicated in the upregulation of several genes involved in erythroid differentiation in cytogenetically normal AML (CN-AML)

patients (Marcucci et al., 2011). Interestingly, high expression of miR-181a has been recently reported to associates with poor prognosis in CN-AML patients (Schwind et al., 2010).

Analysis of human and mouse genomes reveals that miRNA genes are frequently located at fragile sites and regions with CNVs associated with cancer. Moreover, a common mechanism that leads to the deregulation of both the host gene and the co-expressed miRNA has been observed in some tumors (Ambs et al., 2008; Grady et al., 2008). These findings suggest that genomic instability could be an important mechanism of miRNA deregulation in cancer (Calin et al., 2004). Recently, Starczynowsky et al. (2011) revised the relationship between genome-wide alterations and miRNAs deregulation in AML, and found 18 miRNAs which map to leukemia associated CNVs, including miR-143, miR-145, miR-146a, miR-155, miR-181, miR-221 and miR-222, all implicated in cellular processes relevant to AML (Starczynowsky et al., 2011).

Here, we analyzed 16 myeloid cell lines using SNP and mRNA arrays, and obtained the expression pattern of 250 mature miRNAs by real-time PCR (QRT-PCR). This integrative approach, together with bioinformatics and functional studies, showed that CNVs are alternative mechanisms that regulate miRNA expression levels in AML, and allowed the identification of novel candidate genes involved in AML. We found that miR-379 indirectly regulates c-CBL, and that the direct target of miR-370 was *NF1*. Furthermore, analysis of samples of patients with AML at diagnosis suggests that *NF1* downregulation by overexpression of miR-370 could be a recurrent event in AML, allowing distinguishing a subgroup of patients susceptible of targeted therapies.

Materials and methods

Cell culture and transfection

EOL-1, HL-60, Kasumi-1, OCI-AML2, MOLM13, MV4-11, HEL, KG-1, KYO-1, K562 and MEG-01 cells were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS); NOMO-1 and KU-812 in RPMI-1640 with 20% FBS; F-36P in RPMI-1640 with 20% FBS, and 10ng/ml GM-CSF; MUTZ-3 in 80% alpha-MEM with 20% FBS and 10ng/ml GM-CSF; and TF-1 in RPMI-1640 with 20% FBS and 10ng/ml GM-CSF. Cell lines were grown at 37ºC in a 5% CO2 atmosphere. Media were supplemented with penicillin G (100U/ml), and streptomycin (0.1mg/ml). For transfection experiments HL-60 cells were seeded in culture flasks and transfected using the Nucleofector System (solution V and protocol T-019) (Amaxa) with 5nM of pre-miRNAs designed and synthesized by Ambion (Applied Biosystems) (hsa-miR-370 ID/PM12868; hsa-miR-127-3p ID/10400; hsa-miR-432 ID/PM10838; hsa-miR-379 ID/PM10316; hsa-miR-494 ID/PM12409).

Patient samples

The study comprised bone marrow samples of 31 patients with AML at diagnosis. All patients were treated with standard induction chemotherapy. High dose cytarabine, and autologous or allogenic stem cell transplantation when possible, were used as consolidation therapy. Bone marrow samples of normal healthy donors were used as controls. All samples were taken anonymously.

Single Nucleotide Polymorphism Array Analysis

Whole genome analyses were performed using the GeneChip Mapping 500K Array Set (Affymetrix). Genomic DNA samples were isolated using QiAmpDNA MiniKit (Qiagen). Arrays were scanned individually using the GeneChip® Scanner 3000 7G under the GeneChip® CEL files were generated using Affymetrix GeneChip Command Console operating software and Genotyping Console 2.1 according to the manufacturer protocols (Affymetrix). We only analyzed samples which met the quality control (QC) thresholds recommended by Affymetrix in their Genotyping Console v2.1 software. The QC call rate of samples analyzed was at least 96%. Samples not meeting this specification were excluded from further analysis. CEL files were then imported into Partek Genomic Suite and analyzed using the Copy Number Analysis workflow. Regions of CNVs were detected using an unpaired analysis and Genomic segmentation algorithm in the standard Partek. Genomic alterations identified by SNP array (SNPa) were compared with the 500K HapMap Genotype Data Set. We consider as amplification the regions whose copy number was over 3 copies (high amplification CN>5), and as deletion, regions with copy number below 1.5 (homozocigotic deletion CN< 0.5 and hemizygous deletions CN=0.8-1.5) (Schiffman et al., 2009). A region was defined as recurrent alteration when it appears in at least 2 samples. We considerer the karyotype of each cell line to asses that results obtained in the SNPa analysis are not due to the presence of a previously described cytogenetic aberration.

Validation of copy number alterations

Genomic regions located within miRNAs genes were amplified with the primers included in Supplementary Table 1. Glucose-6-Phosphate Dehydrogenase (*G6PDH*), Hydroxymethylbilane Synthase (*HEM3*) and Chloride channel 7 (*CLCN7*) genes were selected as internal controls for varying input DNA amounts as recommended by prior published guidelines (Weksberg et al., 2005; Babashah et al., 2009). Thus, any difference in the real time PCR obtained for test primers/markers would correspond to differences in the amount of the target sequence primers. SYBR Green I real time PCR assays were carried out in final reaction volumes of 15µl with 7.5µl of SYBR Green I Master mix (Applied Biosystems), 1µM of forward and reverse primers and 10ng of genomic DNA. Real time PCR reactions were performed using the 7500 Real Time PCR System (Applied Biosystems). The reaction profile was: initial step, 50°C for 2 min, denaturation, 95°C for 10 min, then 40 cycles of denaturing at 95°C for 15 sec, combined annealing and extension at 60°C for 30 sec and 72ºC 30 sec, followed by the dissociation stage of 72ºC 10 min. Data were analyzed as previously described (Weksberg et al., 2009).

Quantification of miRNA expression levels

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to manufacturer's instructions. cDNA was synthesized with SuperScriptIII Reverse Transcriptase (Invitrogen). Quantification of the expression of *NF1* and c-*CBL* was performed using TaqMan Gene Expression Assays (Applied Biosystems) specific for each gene. GAPDH was used as internal control. For quantification of miRNA expression levels, samples were reverse transcribed using the TaqManHMicroRNA Reverse Transcription Kit (P/N 4366597, Applied Biosystems) and mature miRNAs were quantified by quantitative real-time RT-PCR (QRT-PCR) using the TaqManH MicroRNA Assays - Human Panel Early Access Kit (P/N 4365409, Applied Biosystems). The kit contained assays for 250 miRNAs of the 733 currently listed in the Sanger miRBase database. Expression levels of miR-127, miR-370, miR-379, miR-494, and miR-432 were confirmed using TaqMan MicroRNA Assays (Applied Biosystems) specific for each miRNA and *U6B* as internal control. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001), where $\Delta\Delta C_T = (C_{T,\text{Target Gene}} - C_{T,\text{GAPDH or U6B}})_{\text{Cell Line}} - (C_{T,\text{Target Gene}} - C_{T,\text{GAPDH or U6B}})_{\text{Normal Control}}$.

Gene Expression Profiling

Total RNA was extracted from cell lines using miRNEasy Mini Kit (Qiagen) following manufacturer's protocol. The RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent). Whole genome expression analysis was performed in the cell lines using the Affymetrix Human Genome-U133 Plus-2.0, which contains 54,676 probesets (47,000 transcripts). Microarray data analysis consisted in background correction and normalization using RMA algorithm (Irizarry et al., 2003) and a filtering process to eliminate low expression probesets. LIMMA (Linear Models for Microarray Data) (Smyth, 2004) was used to identify the probesets with significant differential expression. Genes were selected as

significant using a B statistic cut off (B>0) or a less stringent p-value cut off ($p<0.001$).

Integrative Genomic Analyses

Relationship between miRNA expression and the inferred CN of the corresponding miRNA gene was measured by non-parametric analyses. Data from SNP and miRNA analysis were crossed and analyzed using t-tests, and correlations were considered significant when $p<0.05$. The set of differentially expressed genes were then compared with the miRNA predicted targets stored in public databases (Friedman et al., 2009; Lall et al., 2006; Kertesz et al., 2007; Betel et al., 2008; Griffiths-Jones et al., 2008).

Western blot analysis

Cells were lysed in 100µl of lysis buffer containing 1% Triton X-100 and the protease inhibitor cocktail Complete Mini (Roche Diagnostics). After incubation on ice (30 minutes), protein extracts were clarified (12,000xg, 15 minutes, 4°C), denatured and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. Antibodies used were goat polyclonal anti-c-CBL (Cell Signalling), anti-NF1 (Santa Cruz) and mouse monoclonal anti-β-actin (Sigma-Aldrich). Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare).

Luciferase assays

Luciferase assays were done using the Dual Luciferase System (Promega). One hundred nanograms of pRL-NF1(3´UTR), pRL-CBL(3´UTR) or pRL were transfected in the presence of 5 ng of pre-miR-370, pre-miR-379 or pre-miR-negative control, and 50 ng of pGL3-Promoter. A pRL-NF1(3´UTR) construct including a mutated miR-370 seed region was used to confirm *NF1* as a direct target of miR-370. Renilla luciferase activities were normalized to firefly luciferase activities.

Results

Integrative Analysis of Gene Copy Number and miRNA Expression

SNPa analysis in 16 myeloid cell lines showed several genetic aberrations affecting regions previously reported to be involved in AML (Supplementary Table 2). We observed 5 genomic regions with recurrent amplifications and 2 with deletions (present in 4 cell lines or more) (Supplementary Figure 1 and Supplementary Table 3). Moreover, we found 6 high amplifications (CN>5), and 8 homozygous deletions (CN<0.5) (Supplementary Figure 2 and 3, and Supplementary Table 4). Genomic deletions on 9p21 were identified in 9 out of 16 cell lines, and most of these deletions were homozygous (7/9, 77.7%).

Then, we obtained by QRT-PCR the expression profile of 250 miRNAs in these myeloid cell lines, identifying 12 upregulated and one downregulated miRNAs, when comparing to normal control (Table 1). In addition, expression of miR-199b differed significantly between AML and chronic myeloid leukemia in blast crisis (CML-BC) cell lines (data not shown). We next evaluated whether the miRNAs located within the amplified or deleted regions identified by the genome-wide analysis were up- or downregulated. Nineteen miRNAs showed a significant correlation ($p<0.05$) between their expression and the CNV of the genomic region in which they are located: We found 16 miRNAs upregulated and located in two genomic regions of amplification (11q24.1 and 14q32.31), and 3 miRNAs downregulated and located in regions with genomic deletions (miR-15a and miR-16-11, clustered in 13q14.3; and miR-7, located on 9p21.32) (Figure 1). In addition, we detected a cluster on 19q13.41 integrated by 31 miRNAs that were upregulated in 9 myeloid cell lines (data not shown). However, the expression of these miRNAs did not correlate with CN aberrations, indicating that other mechanisms are involved in the transcriptional deregulation of this cluster. Altogether, these results suggest that copy number alteration is a mechanism that could lead to deregulated miRNA expression in AML cells.

Integrative Analysis of miRNA expression and data from mRNA arrays

These results prompted us to analyze the expression levels of the predicted target genes for those 19 miRNAs whose expression correlated with CN alterations. After performing a whole genome expression analysis in the cell lines (Affymetrix Human Genome-U133 Plus-2.0), we obtained a set of candidate genes whose altered expression (B>0) may result from deregulation of 9 out of these 19 miRNAs. The differential expression of these selected 9 miRNAs and their predicted targets were validated by QRT-PCR (Table 2). Our results indicate that CNVs could alter the expression levels of several miRNAs, then modulating the expression of their target genes.

Identification of *NF1* as a target gene of miR-370

Five out of the 9 selected miRNAs had as potential target genes either *NF1* or c-*CBL*, genes with important functions in myeloid neoplasias. Therefore, we decided first to analyze whether miR-127, miR-370, miR-379, miR-432 or miR-

494, all located on 14q32.31, could regulate *NF1* or c-*CBL* (Table 2 and Supplementary Figure 4). The AML cell line HL-60, with low expression of the miRNAs and high expression of their predicted gene targets, was chosen as a cellular model for miRNA overexpression experiments. Analysis by QRT-PCR confirmed overexpression of miR-127, miR-370, miR-379, and miR-494 after transfection with the corresponding pre-miRNAs (Figure 2A). Transfection of premiR-432 could not be optimized.

Western blot analysis showed that NF1 levels decreased after miR-370 overexpression (Figure 2B). Similar results were obtained for c-CBL after miR-379 overexpression (Figure 2C). No changes neither in NF1 or c-CBL levels were observed after ectopic expression of miR-127 and miR-494 (data not shown). Interestingly, transfection of pRL-NF1(3´UTR) in cells ectopically expressing miR-370 showed decreased luciferase reporter activity, indicating that miR-370 binds to 3´UTR of *NF1* negatively regulating its expression. Analysis using the same construct with the seed region of miR-370 mutated did not show changes in luciferase activity, confirming that miR-370 directly binds and negatively regulates *NF1* mRNA (Figure 2D). However, luciferase assays showed that miR-379 is not a direct regulator of c-*CBL*, suggesting that other molecular mechanisms are involved in the decrease of c-CBL levels observed after miR-379 overexpression (Figure 2E).

Analysis of *NF1* downregulation in AML patient samples

To evaluate the clinical relevance of *NF1* and miR-370 in AML, we analyze the prevalence of *NF1* downregulation together with the expression levels of miR-370 in a series of 31 patients with AML at diagnosis. Patient characteristics are presented in Supplementary Table 5. *NF1* was downregulated in 61% cases (19/31), and miR-370 overexpressed in 35% cases (11/31). Interestingly, we observed *NF1* downregulation in 9 out of the 11 cases with miR-370 overexpression, indicating a good correlation between both alterations. Altogether, these results would indicate that *NF1* dowregulation is a recurrent event in AML, and that miR-370 overexpression is an important mechanism to downregulate *NF1* in AML patients.

Discussion

In AML, the association of miRNA expression profiles with cytogenetic or molecular characteristics has been investigated (Marcucci et al., 2009; Marcucci et al., 2011). Deregulation of miRNAs occurs in cancer through different mechanisms (Garzon and Croce, 2008); however, the presence of copy number alterations represents an alternative mechanism poorly studied in AML at present. In this work, we analyze the importance of this mechanism in myeloid leukemia cells using an integrative analysis by SNP and mRNA arrays, together with the quantification of the expression of 250 mature miRNAs. We identified 19 miRNAs with a significant correlation ($p<0.05$) between their expression and the CNV of the genomic region in which they are located (Figure 1), suggesting that copy number alterations could be responsible of the altered expression of several miRNAs in AML cells. To further investigate the biological importance of the alterations observed, we analyzed data from mRNA arrays, identifying that 9 out of the 19 miRNAs altered by the presence of CNVs, had deregulated expression of some of their predicted gene targets (Table 2). This observation would indicate that CNVs can alter the expression of miRNAs, and as a consequence, modulate the expression of their corresponding target genes.

We observed that *NF1* and c-*CBL*, two genes previously implicated in myeloid leukemias, were putative targets of 5 out of the 9 miRNAs upregulated that were located in amplified regions, and showed that miR-370 is a negative regulator of *NF1* (Figure 2). Furthermore, we found that downregulation of *NF1* might be a recurrent event in adult AML cases and that in 47% of these cases the cause could be the overexpression of miR-370. However, 53% of the cases with *NF1* downregulation did not present miR-370 overexpression, suggesting that there are alternative mechanisms to reduce the *NF1* expression in AML.

NF1 encodes neurofibromin, a GTPase-activating protein and negative regulator of Ras (Mullally and Ebert, 2010). Ras pathway activation due to either Ras mutations or upstream gene mutations is common in AML, but knowledge about the role of the tumor suppressor NF1 in AML is still evolving (Parkin et al., 2010). Children with Neurofibromatosis type 1, a dominant familial cancer syndrome, have germline inactivating mutations in *NF1*, and an increased risk of developing juvenile myelomonocytic leukemia (Shannon et al., 1994). However, *NF1* mutations in adult AML seem to be sporadic (Niimi et al., 2006; Parkin et al., 2010). Interestingly, two recent studies have found heterozygous *NF1* deletions in 7% (6/86) (Walter et al., 2009), and 9.5% of adult patients with AML (10/95) (Parkin et al., 2010). Furthermore, Parkin et al. (2010) quantified the expression of *NF1* and provide a complete description of the consequences of *NF1* functional loss in AML, showing that cases with absent NF1 function are sensitive to mTOR inhibition, thus providing a rationale for the study of mTOR inhibitors in *NF1* null AML subsets (Parkin et al., 2010).

As indicated, we found that more than 50% of cases analyzed had *NF1* downregulation. It has been reported that lower *NF1* mRNA expression may contribute to AML pathogenesis, given prior evidence for *NF1* haploinsufficiency in some biological systems (Zhang et al., 2001; Ingram et al., 2000; Chao et al,

2005). The study of Parkin et al. (2010) in AML cases with heterozygous *NF1* deletions and preserved *NF1* expression from the retained allele uncovered some evidence for haploinsufficiency only in the setting of ex vivo blast colony formation; however, the activity of Ras was not substantially influenced in these blasts. Therefore, of importance for our study, they show that cases with *NF1* downregulated had an increase in the proliferation rate of the cells. This is support by previous in vivo studies (Cutts et al., 2009). Altogether this suggests that downregulation of *NF1* is an important event that contributes but it is not sufficient to develop AML.

Here, we showed that miR-370 is a negative regulator of *NF1*, showing a novel mechanism of *NF1* inactivation in AML (Figure 2). Interestingly, in the series of Parkin et al. (2010), 6 AML cases (3 with heterozygous *NF1* deletions and 3 without *NF1* deletions) did not express *NF1* mRNA. Our results suggest that overexpression of miR-370 could be a mechanism contributing to the inactivation of *NF1* in these cases.

MiR-370 is downregulated in malignant cholangiocytes (Meng et al., 2008) and in other gastrointestinal malignancies (Bandres et al., 2006). However, it has been reported the up regulation of a subset of miRNAs, including miR-370, in the human 14q32 imprinted domain in AML patients (Dixon-McIver et al., 2008). Recently, it has been observed that miR-370 expression is activated by c-Jun, playing a role in regulating lipid metabolism (Iliopoulos et al., 2010). Taking into account that SET induces c-Jun activation by inhibiting PP2A (Al-Murrani et al., 1999), increased expression of miR-370 would be expected to play an oncogenic role in the AML model. Furthermore, PP2A inhibition is a recurrent event in AML and that activation of SET, an endogenous PP2A inhibitor, is a mechanism to inactivate PP2A in AML cells (Cristobal et al., 2011). Altogether, these observations suggest that miR-370 could have oncogenic or anti-oncogenic properties depending on the cellular model and molecular background. Therefore, our results provide a novel mechanism to inactivate the tumor suppressor *NF1* in the leukemic cells, in addition to the previously reported chromosomal rearrangements, pointed mutations and microdeletions.

On the other hand, we found a decrease in c-CBL protein levels when we overexpressed the miR-379. Nevertheless, we did not observe a decrease of luciferase reporter activities (Figure 2), indicating that miR-379 regulates indirectly c-CBL, probably trough the regulation of a transcription factor involved in c-CBL regulation. *CBL* includes a group of highly conserved ubiquitin ligases family (E3) that has been involved in human malignancies. *CBL* mutations have been found in a wide variety of myeloid neoplasias including AML (Kales et al., 2010). In addition, CBL can downregulate FLT3, a receptor tyrosine kinase that is frequently mutated in AML (Sargin et al., 2007). Our results indicate that miR-379 deregulation would represent an alternative mechanism to alter c-CBL levels. However, more studies to clarify the mechanism by which miR-379 leads to decreased c-CBL protein levels in AML are needed.

In summary, our integrative analysis including data from high resolution SNPs arrays, mRNA expression arrays, and miRNAs expression profiles in 16 myeloid cell lines allowed us to identify 19 miRNAs whose expression levels significantly correlate with the presence of CNV in the genomic region in which those miRNAs are located. Of relevance, bioinformatics and functional studies

identified *NF1* as a target of the miR-370, reporting a novel mechanism for *NF1* downregulation in AML. Moreover, we observed that miR-379 indirectly regulates c-CBL, decreasing its expression levels. Therefore, our approach showed that CNVs are alternative mechanisms that regulate miRNA expression levels in AML, and allowed the identification of novel candidate genes involved in AML.

Acknowledgements: This work was supported by Ministerio Educación y Ciencia (SAF2005/06425 and AP2006-03038), Ministerio Ciencia e Innovación (PI081687), Gobierno de Navarra (14/2008), ISCIII-RTICC (RD06/0020/0078), and FIMA y UTE (Spain). We thank Alberto Orta for bioinformatics analysis and Marisol Gonzalez-Huarriz for technical assistance.

Authorship Contributions: IC, LGO, CC and MGS performed research; MJC provided samples; IC and MDO designed research and wrote the paper.

Conflict of interest disclosure: No potential conflicts of interest were disclosed.

Tables

Table 1 MiRNAs deregulated in 16 myeloid cell lines.

miRNA	logFC	B value
miR-31	9,52	13,36
miR-150	12,61	8,66
miR-199a	9,86	7,56
miR-26a	4,25	5,11
miR-26b	4,04	3,44
miR-518b	4,63	3,08
miR-23b	3,11	2,79
miR-30a-3p	2,21	1,47
let7g	3,98	1,38
miR-200c	2,19	1,11
miR-145	5,12	1,06
miR-95	4,99	0,22
miR-139	5,11	0,09

Table 2 MiRNAs deregulated by Copy Number alterations that target *NF1* and *CBL*.

MicroRNA	p value*	Putative targets
hsa-miR-127	0,047	*CBL*
hsa-miR-370	0,031	*NF1*
hsa-mir-379	0,000	*CBL*, *NF1*
hsa-miR-432	0,050	*CBL*, *NF1*
hsa-miR-494	0,000	*CBL*, *NF1*

**p-value from correlation analysis between the miRNA expression and the region with CNV*

Figures

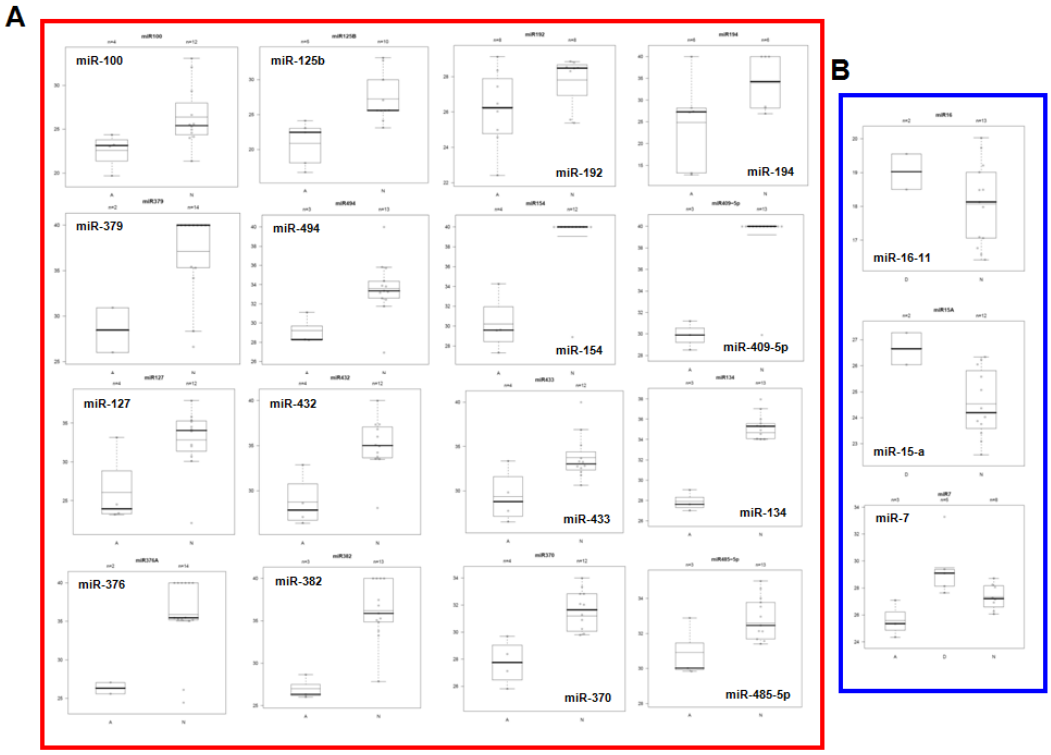


Figure 1. Boxplots of miRNAs whose expression significantly correlated with the inferred CN of the corresponding region. Cell lines are classified as carrying or not a gain/amplification (inferred CN > 3) (A) or a loss/deletion (inferred CN < 1.5) (B) of each specific miRNA gene.

Figure 2

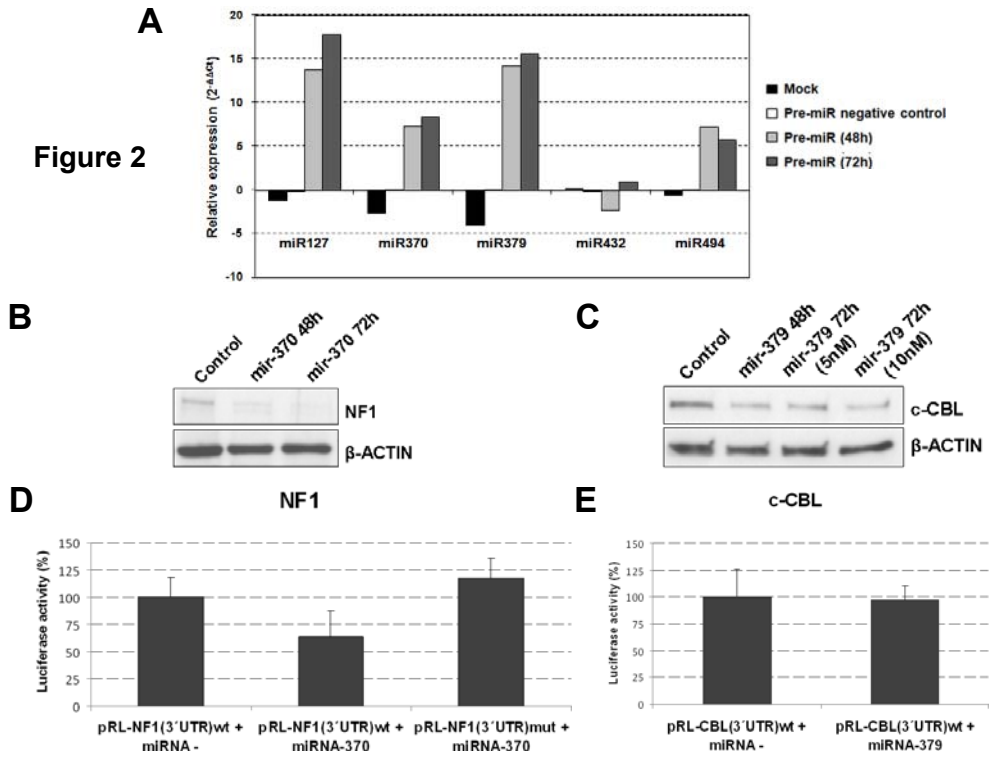


Figure 2. Functional analysis to determine whether miR-127, 370 379, 432 or 494 certainly regulate the candidate target genes *NF1* and c-*CBL*. Analysis by real-time PCR after transfection with pre-miRs-127, 370, 379, 432 and 494 in HL-60 cells (A); Western blot showing NF1 (B) and c-CBL levels (C) after transfection, respectively, with pre-miR-370 and pre-miR-379; Luciferase assay showing changes in luciferase activities after transfection with pre-miR – (negative control) or pre-miR-370 in cells expressing the 3´UTR region of *NF1* that includes the miR-370 seed region [pRL-NF1(3´UTR)wt]. Transfection with the 3´UTR region of *NF1* including a mutated seed region for miR-370 was used as control (D). Luciferase assay after transfection with pre-miR – or pre-miR-379 in cells transfected with the 3´UTR region of c-*CBL* including the seed region for miR-379 (E).

References

Al-Murrani SW, Woodgett JR, Damuni Z. Expression of I2PP2A, an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity. *Biochem J*. 1999;341 (Pt 2):293-298.

Ambs S, Prueitt RL, Yi M, et al. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Res*. 2008;68(15):6162-6170.

Babashah S, Jamali S, Mahdian R, et al. Detection of unknown deletions in beta-globin gene cluster using relative quantitative PCR methods. *Eur J Haematol*. 2009;83(3):261-269.

Bandres E, Cubedo E, Agirre X, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Mol cancer*. 2006;5:29.

Becker H, Marcucci G, Maharry K, et al. Favorable prognostic impact of NPM1 mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2010;28(4):596-604.

Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res*. 2008;36(Database issue):D149-153.

Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*. 2004;101(9):2999-3004.

Chao RC, Pyzel U, Fridlyand J, et al. Therapy-induced malignant neoplasms in Nf1 mutant mice. *Cancer Cell*. 2005;8(4):337-348.

Cutts BA, Sjogren AK, Andersson KM, et al. Nf1 deficiency cooperates with oncogenic K-RAS to induce acute myeloid leukemia in mice. *Blood*. 2009;114(17):3629-3632.

Dixon-McIver A, East P, Mein CA, et al. Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia. *PLoS One*. 2008;3(5):e2141.

Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19(1):92-105.

Garzon R, Croce CM. MicroRNAs in normal and malignant hematopoiesis. *Curr Opin Hematol*. 2008;15(4):352-358.

Garzon R, Garofalo M, Martelli MP, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A*. 2008;105(10):3945-3950.

Grady WM, Parkin RK, Mitchell PS, et al. Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. *Oncogene*. 2008;27(27):3880-3888.

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res*. 2008;36(Database issue):D154-158.

Iliopoulos D, Drosatos K, Hiyama Y, Goldberg IJ, Zannis VI. MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism. *J Lipid Res*. 2010;51(6):1513-1526.

Ingram DA, Yang FC, Travers JB, et al. Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumor suppressor gene modulates melanocyte and mast cell fates in vivo. *J Exp Med*. 2000;191(1):181-188.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*. 2003;31(4):e15.

Kales SC, Ryan PE, Nau MM, Lipkowitz S. Cbl and human myeloid neoplasms: the Cbl oncogene comes of age. *Cancer Res*. 2010;70(12):4789-4794.

Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in microRNA target recognition. *Nat Genet*. 2007;39(10):1278-1284.

Lall S, Grun D, Krek A, et al. A genome-wide map of conserved microRNA targets in C. elegans. *Curr Biol*. 2006;16(5):460-471.

Li Z, Lu J, Sun M, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. *Proc Natl Acad Sci U S A*. 2008;105(40):15535-15540.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.

Loh ML. Childhood myelodysplastic syndrome: focus on the approach to diagnosis and treatment of juvenile myelomonocytic leukemia. *Hematology Am Soc Hematol Educ Program*. 2010;2010:357-362.

Marcucci G, Mrozek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. *Blood*. 2011;117(4):1121-1129.

Marcucci G, Radmacher MD, Mrozek K, Bloomfield CD. MicroRNA expression in acute myeloid leukemia. *Curr Hematol Malig Rep*. 2009;4(2):83-88.

Meng F, Wehbe-Janek H, Henson R, Smith H, Patel T. Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes. *Oncogene*. 2008;27(3):378-386.

Mullally A, Ebert BL. NF1 inactivation revs up Ras in adult acute myelogenous leukemia. *Clin Cancer Res*. 2010;16(16):4074-4076.

Niimi H, Harada H, Harada Y, et al. Hyperactivation of RAS signaling pathway in myelodysplastic syndrome with AML1/RUNX1point mutations. *Leukemia*. 2006;20(4):635-644.

O'Connell RM, Rao DS, Chaudhuri AA, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med*. 2008;205(3):585-594.

Parkin B, Ouillette P, Wang Y, et al. NF1 inactivation in adult acute myelogenous leukemia. *Clin Cancer Res*. 2010;16(16):4135-4147.

Sargin B, Choudhary C, Crosetto N, et al. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. *Blood*. 2007;110(3):1004-1012.

Schiffman JD, Wang Y, McPherson LA, et al. Molecular inversion probes reveal patterns of 9p21 deletion and copy number aberrations in childhood leukemia. *Cancer Genet Cytogenet*. 2009;193(1):9-18.

Schwind S, Maharry K, Radmacher MD, et al. Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2010;28(36):5257-5264.

Shannon KM, O´Connell P, Martin GA, et al. Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *N Engl J Med*. 1994;330(9):597-601.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.

Starczynowski DT, Morin R, McPherson A, et al. Genome-wide identification of human microRNAs located in leukemia-associated genomic alterations. *Blood*. 2011;117(2):595-607.

Waldman SA, Terzic A. A study of microRNAs in silico and in vivo: diagnostic and therapeutic applications in cancer. *FEBS J*. 2009;276(8):2157-2164.

Walter MJ, Payton JE, Ries RE, et al. Acquired copy number alterations in adult acute myeloid leukemia genomes. *Proc Natl Acad Sci U S A*. 2009;106(31):12950-5.

Weksberg R, Hughes S, Moldovan L, Bassett AS, Chow EW, Squire JA. A method for accurate detection of genomic microdeletions using real-time quantitative PCR. *BMC Genomics*. 2005;6:180.

Yendamuri S, Calin GA. The role of microRNA in human leukemia: a review. *Leukemia*. 2009;23(7):1257-1263.

Zhang Y, Taylor BR, Shannon K, Clapp DW. Quantitative effects of Nf1 inactivation on in vivo hematopoiesis. *J Clin Invest*. 2001;108(5):709-715.

Supplementary Information

Supplementary Table 1 The oligonucleotide primers used for real-time PCR in the CN validation process.

Primer Name	Primer sequence	Chromosome	Amplicon size (bp)
G6PDH_Fwd	TCTTCATCACCACAGAGAACT	1	221
G6PDH_Rev	GACCTGGAAGTCACTGGGCA		
HEM3_Fwd	TGCACGGCAGCTTAACGAT	11	202
HEM3_Rev	AGGCAAGGCAGTCATCAAG		
CLCN7_Fwd	CTCTTAGGCCAGGCGTTTGTG	16	128
CLCN7_Rev	ACCGTGCTCAGCGCTATGC		
miR-15/16_Fwd	TTTATATTCTTTAGGCG	13	291
miR-15/16_Rev	AACACAACTGTAGAGTAT		
miR-125a_Fwd	TGCTTTGTCTCAAGAA	11	198
miR-125a_Rev	TACTCAATCACCTCAG		
miR-100_Fwd	ATGTCACAGCCCCAAAA	11	131
miR-100_Rev	CCAGGTCCGTGAGATTG		
miR-379_Fwd	GTCAGCACCATTCCGTG	14	115
miR-379_Rev	CCAGGTCCGTGAGATTG		
miR-494_Fwd	GAATCTTCCTGGAGGT	14	276
miR-494_Rev	GCATGGCACGCTGTCA		
miR-370_Fwd	TACAAGTCGGGGCACA	14	107
miR-370_Rev	CTGGTGTTAGACAGAC		
miR-127_Fwd	GATGGGTTGACTGATGC	14	164
miR-127_Rev	CGTCGGGCAGTGGAGTG		
miR-432_Fwd	GATGGGTTGACTGATGC	14	280
miR-432_Rev	CGTCGGGCAGTGGAGTG		

Supplementary Table 2 Genomic regions previously described to be implicated in AML.

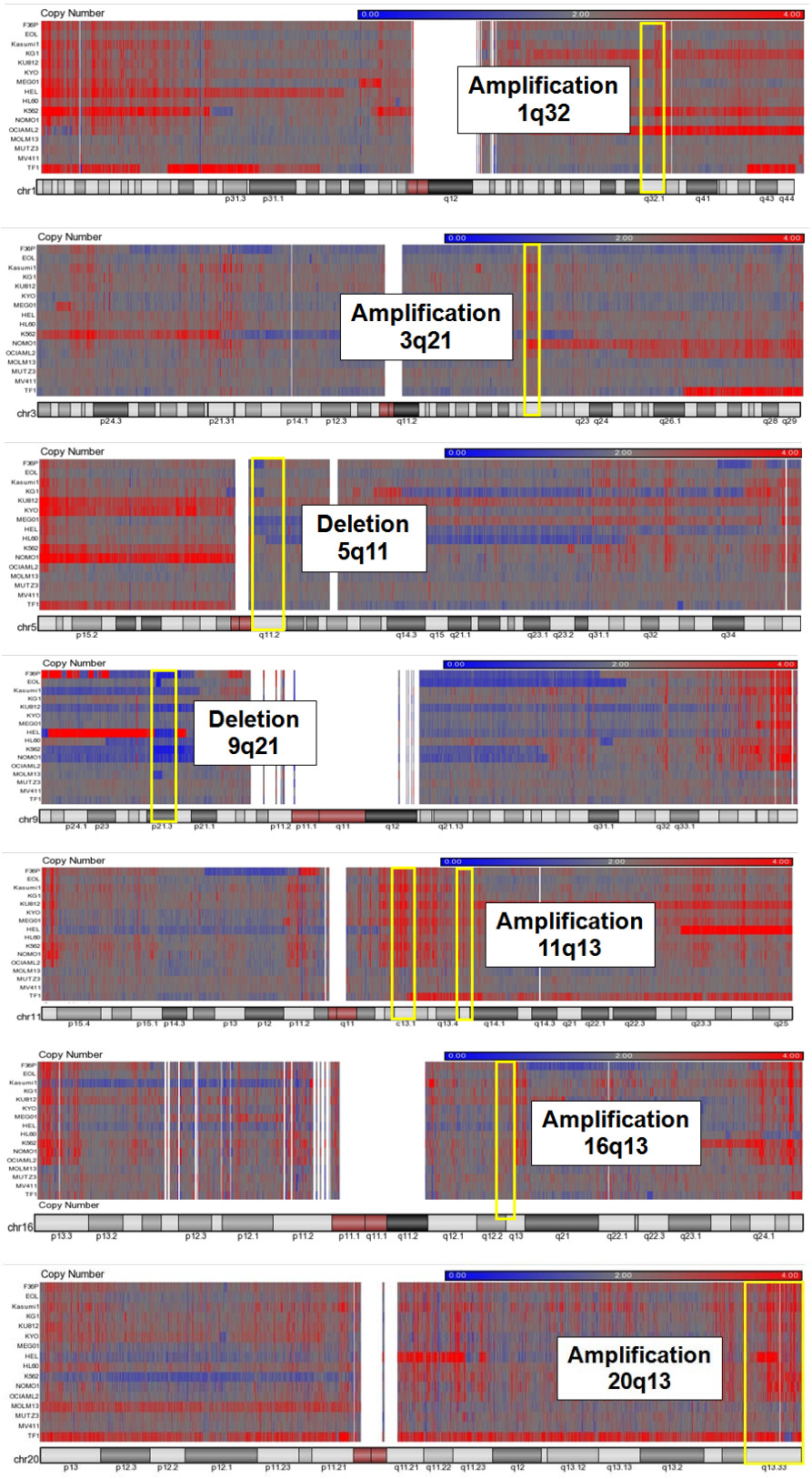
Location	Status	Paper
3p14.1	deletion	(Walter et al., 2009)
5p14.3-q11.2	amplification	(Akagi et al., 2008)
5p15.31	amplification	(Akagi et al., 2008)
5q31.1	deletion	(Walter et al., 2009)
7p21.3	deletion	(Radtke et al., 2009; Asou et al., 2009)
8q24.13-q24.21	amplification	(Akagi et al., 2008; Radtke et al., 2009)
11p14.1	deletion	(Radtke et al., 2009)
11q23.3	amplification	(Walter et al., 2009)
12p12.3	deletion	(Walter et al., 2009)
12p13.31-p13.2	deletion	(Akagi et al., 2008)
16q22.1	deletion	(Radtke et al., 2009)
19p13.2	amplification	(Radtke et al., 2009)
21q21.2	deletion	(Akagi et al., 2008)
21q22.2	amplification	(Walter et al., 2009; Radtke et al., 2009.)

Supplementary Table 3 Genomic regions with recurrent amplifications or deletions. Amplification was considered if CN > 3, and deletion if CN < 1.5; A: amplification; D: deletion.

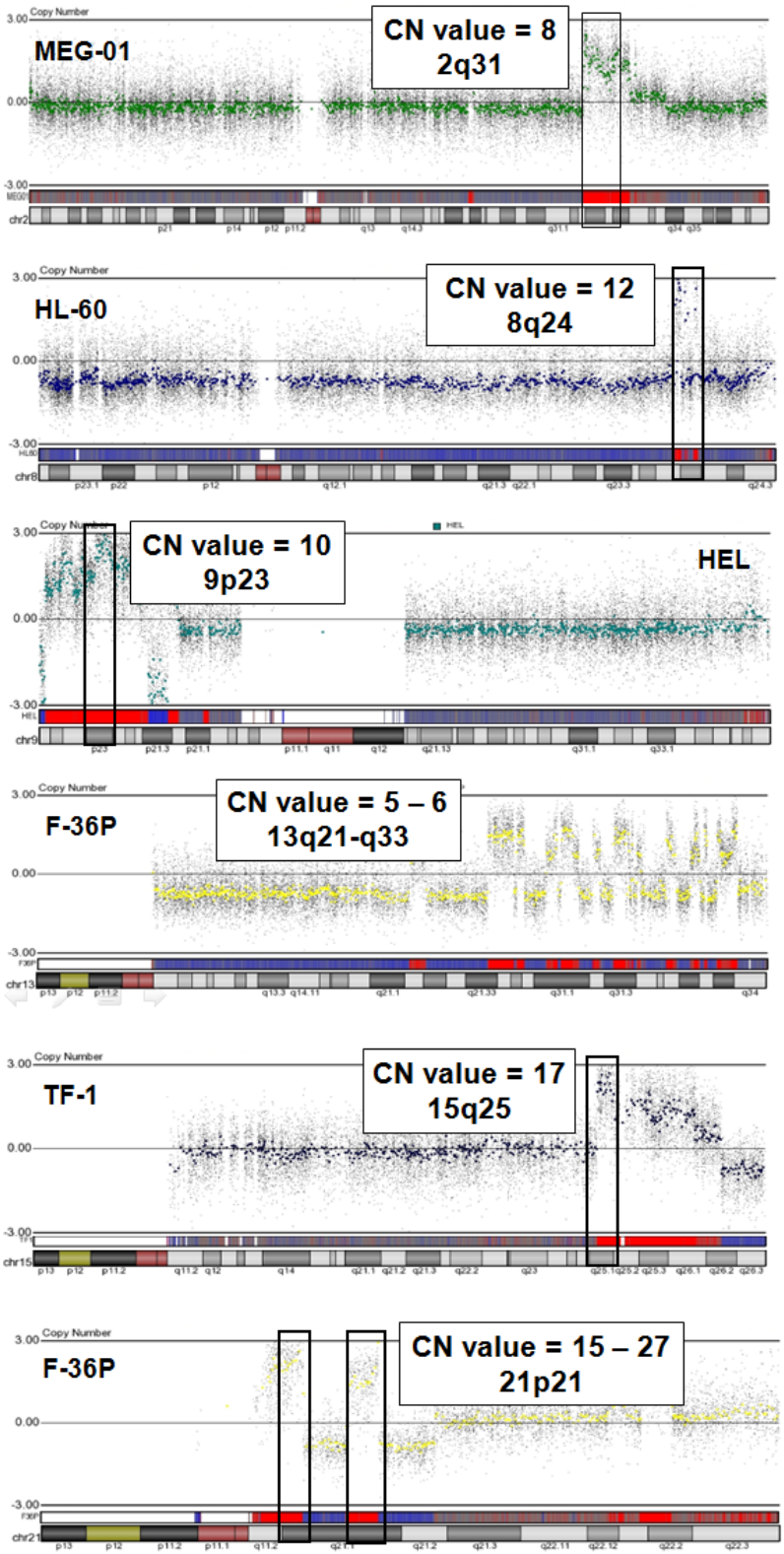
	Cell line	Cytoband	Start	End	Length (bps)	Copy number value	Markers
A	EOL-1	1q32.1	201571229	201606873	35644	3,18	28
	Kasumi-1	1q32.1	203711819	203752179	40360	4,45	15
	KU-812	1q32.1	199892235	199924702	32467	4,38	10
	MEG-01	1q32.1	199892235	199924702	32467	3,91	10
	K562	1q32.2 - q42.1	199100957	223701729	24600772	3,56	4837
	NOMO-1	1q32.1	203232845	203253176	20331	4,14	13
	MOLM13	1q32.1	201415031	201439334	24303	3,42	10
A	EOL-1	3q21.2	127326135	127350448	24313	4,86	10
	Kasumi-1	3q21.2 - 3q21.3	127326135	128259831	933696	6,27	20
	HEL	3q21.2 - 3q21.3	127326135	129002970	1676835	3,60	193
	NOMO-1	3q21.2 - 3q21.3	127428799	165271748	37842949	3,66	5926
	OCI-AML2	3q21.2 - 3q21.3	127326135	128259831	933696	4,22	20
A	KYO-1	11q13.1	63637120	63855796	218676	3,39	20
	K562	11q12.2 - q13.5	60265161	75596147	15330986	3,38	1865
	NOMO-1	11q13.1	63628813	63684339	55526	4,55	10
	KU-812	11q13.1 - q13.5	63725602	75596147	11870545	3,82	1422
	F-36P	11q13.5	75544219	75596147	51928	4,17	15
	EOL-1	11q13.5	75544219	75596147	51928	3,88	15
	Kasumi-1	11q13.5	75544219	75596147	51928	4,61	15
	KG-1	11q13.5	75567031	75644651	77620	3,94	11
	MEG-01	11q13.5	75567031	75644651	77620	5,30	11
	HEL	11q13.5	75544219	75584526	40307	4,05	13
	OCI-AML2	11q13.5	75544219	75584526	40307	3,68	13
A	KG-1	16q13	55431557	55496746	65189	3,94	11
	KYO-1	16q13	55431557	55496746	65189	3,72	11
	MEG-01	16q13	55431557	55498995	67438	3,35	13
	NOMO-1	16q13 - q21	56561169	56714732	153563	3,62	13
A	Kasumi-1	20q13.33	58939610	58965922	26312	4,84	12
	HEL	20q13.33	58845445	60466282	1620837	3,89	365
	HL-60	20q13.33	61458210	61551456	93246	4,36	10
	TF1	20q13.33	61634576	62376959	742383	3,18	69
D	F-36P	5q11.1 - 5q11.2	48007854	53267246	5259392	1,14	558
	MEG-01	5q11.1 - 5q12.3	48007854	64751326	16743472	1,27	2772
	MOLM13	5q11.2	54114560	54140718	26158	1,04	10
	HL-60	5q11.2 - 5q15	55915416	94330201	38414785	1,26	6076
D	F-36P	9p21.3	21437624	25424830	3987206	0,37	762
	EOL-1	9p21.3	21253887	22151353	897466	0,30	214
	KU-812	9p21.3	20928278	25162803	4234525	0,69	787
	HEL	9p21.3	20969884	24744643	3774759	0,36	704
	K562	9p21.3	20735106	24705687	3970581	0,37	741
	NOMO-1	9p21.3	20461117	25147125	4686008	0,37	884
	MOLM13	9p21.3	20438426	22411876	1973450	0,75	422

Supplementary Table 4 List of the high amplifications (CN > 5) and homocigotic deletions (CN < 0.5) found in the copy number analysis of 16 myeloid cell lines.

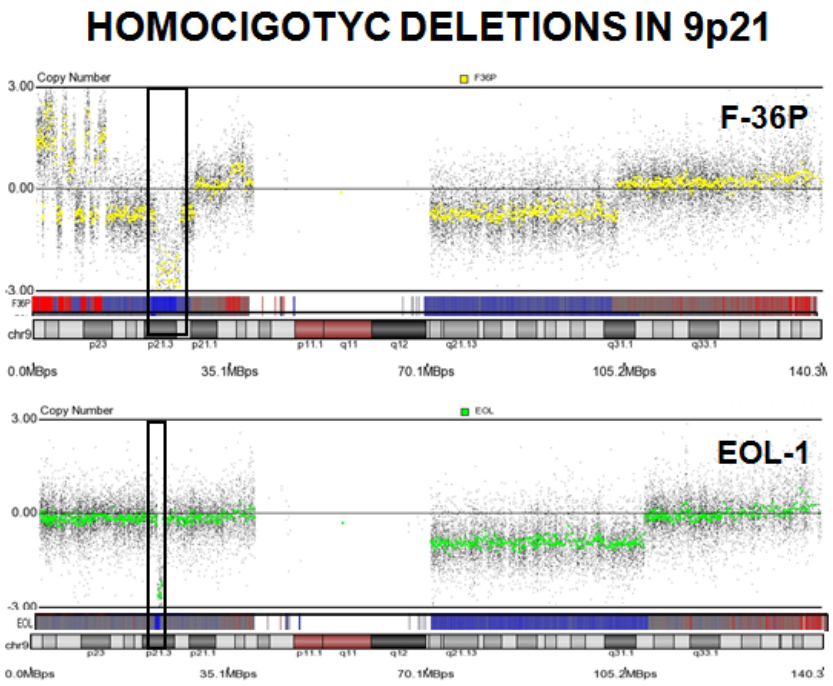
HIGH AMPLIFICATIONS	Cell line	start	end	Cytoband	Length (bps)	Copy Number value	Markers
Chromosome 2	**MEG-01**	182331491	182626451	2q31.3	294960	8,37	38
	MEG-01	182736516	183111418	2q32.1	374902	8,48	81
Chromosome 8	**HL-60**	130064526	130273204	8q24.21	208678	12,81	29
	MEG-01	132931702	132995383	8q24.22	63681	12,09	11
Chromosome 9	**F-36P**	9174669	9452892	9p23	278223	8,95	89
	F-36P	2446040	2970007	9p24.2	523967	8,97	164
	HEL	11306176	11390166	9p23	83990	10,32	17
Chromosome 13	**F-36P**	70434212	71570951	13q21.33	1136739	5,04	202
	F-36P	81888523	83664992	13q31.1	1776469	5,61	299
	F-36P	86842695	87159117	13q31.2	316422	5,20	33
	F-36P	92221989	92381645	13q31.3	159656	5,66	31
	F-36P	94778104	94828355	13q32.1	50251	6,30	11
	F-36P	98321861	98669537	13q32.3	347676	5,25	70
	F-36P	102720811	103149660	13q33.1	428849	5,02	144
Chomosome 15	**TF1**	77804928	77828350	15q25.1	23422	17,69	10
Chromosome 21	**F-36P**	16507771	16586485	21q21.1	78714	27,37	25
	F-36P	21341239	21432066	21q21.1	90827	15,72	24
HOMOCIGOTIC DELETIONS	**Cell line**	**start**	**end**	**Cytoband**	**Length (bps)**	**Copy Number value**	**Markers**
Chromosome 2	**EOL-1**	50629096	50933139	2p16.3	304043	0,32	59
Chromosome 3	**K562**	60583442	60639341	3p14.2	55899	0,42	10
Chromosome 7	**OCI-AML2**	69369397	69498154	7q11.22	128757	0,29	14
Chromosome 9	**F-36P**	21437624	25773958	9p21.3 - 9p21.2	4336334	0,29	686
	EOL-1	21253887	22151353	9p21.3	897466	0,30	273
	KU-812	21737737	28084024	9p21.3 - 9 p 21.2	6346287	0,30	139
	MOLM13	21935211	22009401	9p21.3	74190	0,35	15
	NOMO-1	20461117	31453529	9p21.3 - 9p21.1	10992412	0,28	1118
	HEL	20969884	24744643	9p21.3	3774759	0,27	613
	HEL	240000	1240626	9p24.3	9p24.3	0,24	161
Chromosome 10	**HL-60**	9066570	9105880	10p14	39310	0,3189	10
	HL-60	21849531	22187383	10p12.31	337852	0,3964	10
	K562	103607635	103739905	10q24.32	132270	0,3932	14



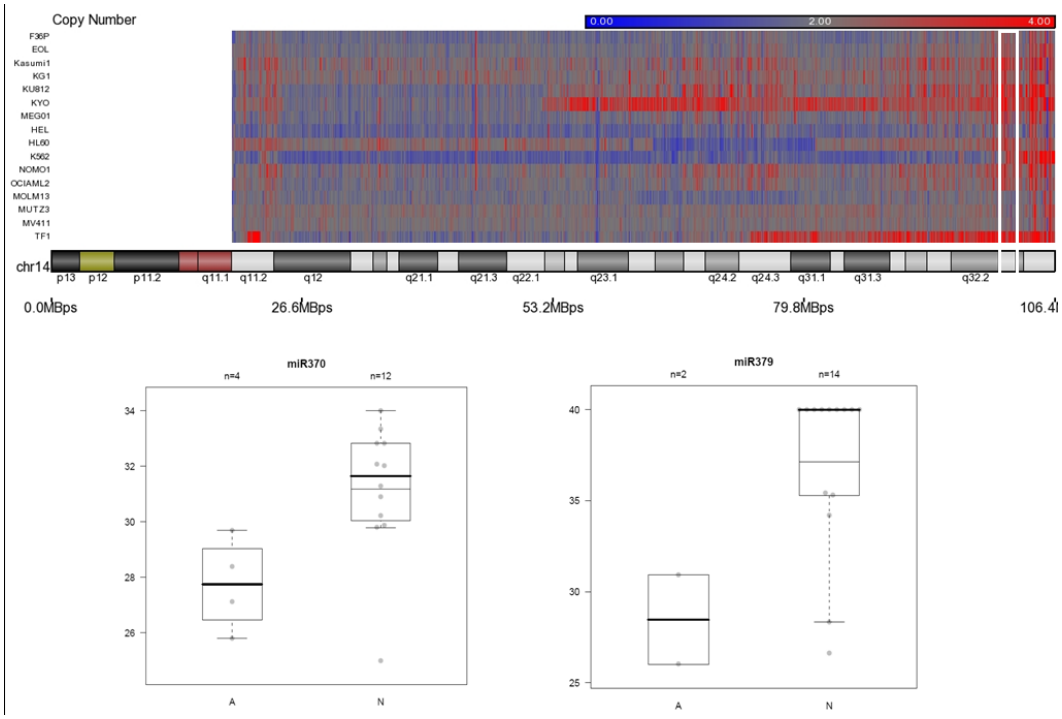
Supplementary Figure 1. Genomic regions including recurrent amplifications or deletions (at least present in 4 cell lines).



Supplementary Figure 2. Images of 6 high amplifications (copy number >5), plotted logarithmically.



Supplementary Figure 3. Images of deletions in 9p21.



Supplementary Figure 4. Genomic region of amplification located in 14q32.31, and box-plots of miR-370 and miR-379, whose expression significantly correlated with the inferred CN of the corresponding region 14q32.31.

<u>Capítulo 2.</u> PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent antileukemic effect

(artículo publicado en la revista *Leukemia*)

Leukemia (2011), 1–9

www.nature.com/leu

ORIGINAL ARTICLE

PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect

I Cristóbal[1,2], L Garcia-Orti[1], C Cirauqui[1], MM Alonso[1], MJ Calasanz[2] and MD Odero[1,2]

[1]*Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Navarra, Spain and* [2]*Department of Genetics, University of Navarra, Pamplona, Navarra, Spain*

PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent antileukemic effect

Ion Cristóbal, BSc[1,2], Laura Garcia-Orti, BSc[1], Cristina Cirauqui, BSc[1], Marta Alonso, PhD[1], María J. Calasanz, PhD[2], María D. Odero, MD,PhD[1,2]

[1]Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain.

[2]Department of Genetics, University of Navarra, Pamplona, Spain.

Running Title: PP2A inhibition in AML

Correspondence: María D. Odero, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, C/ Pio XII-55 31008-Pamplona, Spain. E-mail: modero@unav.es. Phone: +34-948-194700. Fax: +34-948-194714.

Abstract

Protein phosphatase 2A (PP2A) is a human tumor suppressor that inhibits cellular transformation by regulating the activity of several signaling proteins critical for malignant cell behavior. PP2A has been described as a potential therapeutic target in chronic myeloid leukemia, Philadelphia-chromosome-positive acute lymphoblastic leukemia, and B-cell chronic lymphocytic leukemia. Here, we show that PP2A inactivation is a recurrent event in acute myeloid leukemia (AML), and that restoration of PP2A phosphatase activity by treatment with forskolin in AML cells blocks proliferation, induces caspase-dependent apoptosis, and affects AKT and ERK1/2 activity. Moreover, treatment with forskolin had an additive effect with Idarubicin and Ara-c, drugs used in standard induction therapy in AML patients. Analysis at protein level of the PP2A activation status in a series of patients with AML at diagnosis showed PP2A hyperphosphorylation in 78% of cases (29/37). In addition, we found that either deregulated expression of the endogenous PP2A inhibitors SET or CIP2A, overexpression of SETBP1, or downregulation of some PP2A subunits, might be contributing to PP2A inhibition in AML. In conclusion, our results show that PP2A inhibition is a common event in AML cells and that PP2A activators, such as forskolin or FTY720, could represent potential novel therapeutic targets in AML.

Keywords: PP2A, inhibition, AML, therapy

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disease that disrupts normal hematopoiesis. Leukemic cells are characterized by a block in differentiation and apoptosis, together with an enhanced proliferation. Despite progressive advances in our understanding of the molecular biology of AML, patient outcomes are still very poor. Complete remission occurs in up to half of these patients; however, relapse is generally expected and prognosis is dismal.[1] Therefore, it is necessary to develop more effective treatment strategies to improve the survival of these patients.[2]

The unrestricted growth of transformed cells is caused by the cumulative deregulation of multiple cellular pathways involved in normal growth control.[3] The ubiquitously expressed protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase that accounts for most of the serine/threonine phosphatase activity in eukaryotic cells, and participates in many mammalian signaling pathways.[4] PP2A represents a family of heterotrimeric holoenzyme complexes consisting of an active core composed of the scaffold PP2A-A subunit, the catalytic PP2A-C subunit, and a regulatory PP2A-B subunit. There are two closely related isoforms of the PP2A-A (Aα/PPP2R1A and Aβ/PPP2R1B),[5-6] and of the PP2A-C (Cα/PPP2CA and Cβ/PPP2CB) subunits.[7-8] The scaffold subunit mediates interaction of the core dimer with a wide variety of regulatory B subunits that regulate both the specific substrate and the localization of the holoenzyme. Four unrelated families of regulatory B subunits have been identified, including at least 26 different alternative transcripts and splice forms.[9] Therefore, PP2A has the ability to form complexes with many different substrates.[3,10] A variety of mechanisms that inhibit PP2A are present in transformed cells, including alterations in structural or regulatory PP2A subunits, and also the overexpression of specific endogenous inhibitors.[3,10] Somatic mutations of the PP2A structural subunits Aα and Aβ have been described in several types of cancer, causing a defective binding of the B and C subunits and thus inhibiting PP2A activity.[11-20] Moreover, suppression of PP2A Aβ expression permits immortalized human cells to achieve a tumorigenic state through the deregulation of RaIA GTPase activity. Cancer-associated Aβ mutants fail to reverse this tumorigenic phenotype, indicating that these mutants function as null alleles.[21] In addition, both Aα mutants and Aα downregulation lead to a functional haploinsufficiency that seems to induce human cell transformation by activating AKT/PI3K signaling pathway.[22-23] However, it is likely that different sets of genetic aberrations during tumor formation require the loss of different PP2A holoenzyme complexes for the tumor progression, and this would involve the regulatory subunits that are playing a key role directing PP2A to dephosphorylate and regulate key tumor suppressors or oncogenes.[9] In this regard, several members of the B56 family of regulatory PP2A subunits appear to have a main role in directing PP2A potential tumor-suppressive activity.[24-30]

With regard to the endogenous PP2A inhibitors, upregulation of SET by the BCR-ABL oncogene leads to the suppression of PP2A, and contributes to leukemogenesis in chronic myeloid leukemia (CML) and Philadelphia-chromosome positive acute lymphoblastic leukemia (ALL).[31-32] In addition, Junttila et al. (2007) provide strong evidence that CIP2A (cancerous inhibitor of

PP2A) selectively targets PP2A associated with c-Myc to inhibit its phosphatase activity and protect Ser62 from dephosphorylation. Interestingly, CIP2A expression is upregulated in transformed cell lines and cancer tissue samples.[33] Finally, it has been reported that JAK2 directly phosphorylate PP2A at tyrosine 307 of its catalytic subunit, making PP2A inactive.[34]

Few studies have investigated the role of PP2A in AML. Gallay et al. reported that the intensity of phospho-Akt on Thr308 in AML was significantly correlated with high-risk cytogenetics, particularly with a complex karyotype, and they found correlation between decreased PP2A activity and Thr308 phosphorylation in this subgroup (7 cases).[35] Moreover, a recent study shows that activating c-KIT mutations inhibit PP2A, and that reactivation of PP2A effectively suppresses the in vitro and in vivo growth of imatinib-sensitive and imatinib-resistant c-KIT positive cells, indicating that functional inactivation of PP2A tumor suppressor activity could represent a key step in the induction and maintenance of KIT positive leukemias.[36] Our group has previously reported that SETBP1 overexpression is a recurrent event in AML, which impairs PP2A activity via SET and promotes proliferation of AML cells.[37] In addition, it has been reported that the activity of PP1 and PP2A is enhanced in the arsenic sulphide-induced differentiation of the AML cell line HL-60,[38] and that the alkylphosphocholine erucylphosphohomocholine is cytotoxic to AML cells through JNK and PP2A-dependent mechanisms.[39] Interestingly, PP2A activators such as FTY720 in CML, ALL and chronic lymphocytic leukemia (CLL), and forskolin in CML show promising antileukemic effects in both in vitro and in vivo models.

In this study, we show that PP2A activity is reduced in both myeloid Ph negative cell lines and AML patient samples. Treatment with the PP2A activator forskolin restores PP2A activity, affecting proliferation and inducing changes in the phosphorylation status of AKT and ERK1/2. Moreover, we found an additive effect between PP2A activation by forskolin and the chemotherapy reagents Idarubicin and cytosine arabinoside (Ara-c), suggesting that treatment with PP2A activators could be a therapeutic target in AML in combination with standard induction therapy. Finally, deregulated expression of endogenous PP2A inhibitors, together with aberrations affecting the expression of PP2A subunits, were identified as possible mechanisms of PP2A inhibition in AML.

Materials and methods

Cell cultures

EOL-1, HL-60, Kasumi-1, OCI-AML2, MOLM13, MV4-11, HEL, KG-1, KYO-1, K562 and MEG-01 cells were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS); NOMO-1 and KU-812 in RPMI-1640 with 20% FBS; F-36P in RPMI-1640 with 20% FBS, and 10 ng/ml GM-CSF; UT-7 in alpha-MEM (Invitrogen) with 20% FBS and 5 ng/ml GM-CSF; MUTZ-3 in 80% alpha-MEM with 20% FBS and 10 ng/ml GM-CSF; and TF-1 in in RPMI-1640 with 20% FBS and 10 ng/ml GM-CSF. Cell lines were grown at 37ºC in a 5% CO2 atmosphere. Media were supplemented with penicillin G (100 U/ml), and streptomycin (0.1 mg/ml). Cells were treated with the following reagents: Idarubicin (15 nM) (Sigma-Aldrich), Ara-c (2,5 μM) (Sigma-Aldrich), forskolin (40 μM) (Calbiochem), FTY720 (10 μM) (Calbiochem), Z-VAD-fmk (150 mM) (Promega) and okadaic acid (2.5nM) (Calbiochem).

Patient samples

The study comprised bone marrow samples of 37 patients with AML at diagnosis. All patients were treated with standard induction chemotherapy. High dose cytarabine, and autologous or allogenic stem cell transplantation when possible, were used as consolidation therapy. Bone marrow samples of normal healthy donors were used as controls. All samples were taken anonymously.

Nucleic acid isolation and real-time RT-PCR

Total RNA was isolated using the RNeasy minikit (Qiagen). cDNA was synthesized with SuperScriptIII Reverse Transcriptase (Invitrogen). Quantification of the expression of *SETBP1*, *SET*, *CIP2A*, PP2A catalytic subunits alpha (*PPP2CA*) and beta (*PPP2CB*), PP2A scaffold subunit *PPP2R1B*, and PP2A regulatory subunits *PPP2R5B* and *PPP2R5C* was performed using TaqMan Gene Expression Assays (Applied Biosystems) specific for each gene. *GAPDH* was used as internal control. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C}{}_{T}$ method,[40] where $\Delta\Delta C_T = (C_{T,\text{Target Gene}} - C_{T,\text{GAPDH}})_{\text{Cell Line}} - (C_{T,\text{Target Gene}} - C_{T,\text{GAPDH}})_{\text{Normal Control}}$. A gene was considered deregulated if its expression value was higher or lower than the cut-off value established for each gene (mean+3SD), defined by the analysis of 10 normal BM samples.

Western blot analysis

Cells were lysed in 100μL of Lysis buffer containing 1% Triton X-100 and the protease inhibitor cocktail Complete Mini (Roche Diagnostics). After incubation on ice (30min) protein extracts were clarified (12,000 xg, 15min, 4ºC), denatured and subjected to SDS-PAGE and Western-blot. Antibodies used were mouse monoclonal anti-PP2A (clone 1D6, Upstate Inc.), rabbit monoclonal anti-PP2AY307 (Epitomics), rabbit polyclonal anti-Akt, rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology Inc.), rabbit polyclonal anti-pAkt^{Thr308}, rabbit polyclonal anti-pERK1/2$^{Thr202/Tyr204}$ (Santa Cruz Biotechnology), mouse monoclonal anti-βactin (Sigma), rabbit polyclonal anti-CIP2A (Novus

Biologicals), goat polyclonal anti-SET (Santa Cruz Biotechnology), goat monoclonal anti-PPP2R5B (Novus Biologicals) and rabbit polyclonal anti-PPP2R1B (Novus Biologicals). Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare).

Proliferation assay and cell viability

Cell proliferation was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) and following the manufacturer´s indications. Results of cell viability were confirmed by the Trypan Blue dye exclusion test.

PP2A phosphatase activity assays

PP2A assays were performed with cell lysates (50µg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described.[37]

Analysis of apoptosis and caspase activation

Caspase 3/7 activities were measured on untreated and forskolin-treated cells using the caspase Glo-3/7 assay kit (Promega Corp.). Briefly, 5×10^3 cells were plated in a white-walled 96-well plate, and the Z-DEVD reagent, the luminogenic caspase 3/7 substrate containing a tetrapeptide Asp-Glu-Val-Asp, was added with a 1:1 ratio of reagent to sample. After 90 minutes at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal was measured by a FLUOstar OPTIMA luminometer (BMG Labtech). Differences in caspase-3/7 activity in forskolin-treated cells compared with untreated cells are expressed as fold-change in luminescence. Apoptosis was measured using the Annexin-V-FLUOS Staining Kit (Roche) and following manufacturer´s instructions.

Statistical analysis

Data represented are mean of three independent experiments ± s.d. Statistical comparisons were carried out by T-test analysis and significance was considered when $P < 0.05$. Chou-Talalay analysis was performed using the CalcuSyn Software (Biosoft, Cambridge, UK) to determine additivity between forskolin and Idarrubicin/Ara-c treatments.

Microarray analysis

RNA samples were processed following manufacturer protocols (Affymetrix) and hybridized to the Affymetrix Human Genome-U133 Plus-2.0, which contains 54,676 probesets (47,000 transcripts). Microarray data analysis consisted in background correction and normalization using RMA algorithm[41] and a filtering process to eliminate low expression probesets. LIMMA (Linear Models for Microarray Data)[42] was used to identify the probesets with significant differential expression. Samples were distributed in three different groups: “Control” including 3 normal control samples, “AML” including the AML cell lines EOL-1, HL-60, Kasumi-1, OCI-AML2, MOLM13, MV4-11, HEL, KG-1, NOMO-1, F-

36P and TF-1, and “CML” including the CML in blast crisis (BC-CML) cell lines KU-812, KYO-1, K562 and MEG-01. Data represented are the gene expression mean of each group ± s.d. Genes were selected as significant between the different groups using a B statistic cut off (B>0) or a less stringent adjusted *P*-value cut off ($P < 0.05$).

Results

PP2A is inactivated in AML cell lines

Phosphorylation of tyrosine-307 is responsible for more than 90% of the phosphatase activity of PP2A. Moreover, it has been shown that this phosphatase is inactive when tyrosine-307 is phosphorylated.[43] Thus, we assessed the phosphorylation levels on tyrosine-307 of PP2A by western blot in a panel of AML BCR-ABL negative cell lines. Eleven out of 13 cell lines presented increased phosphorylation in this tyrosine. Notably, the two cell lines (Kasumi-1 and MUTZ-3) that had reduced PP2A phosphorylation were the only ones with reduced expression of the catalytic subunit (PP2A-C) (Figure 1). Four BCR-ABL positive CML cell lines, including K562, were used as positive controls for phosphorylation on PP2A tyrosine-307.[31] In order to confirm whether the differences observed in PP2A protein expression in Kasumi-1 and MUTZ-3 were due to reduced transcriptional levels of PP2A, we analyzed by real-time PCR (QRT-PCR) the expression of the PP2A catalytic subunits PPP2CA and PPP2CB separately (Supplementary Table 1). We found no significant differences in PP2A-C transcriptional levels in these two cell lines, indicating that differences observed in western blot must be due to post-transcriptional regulation.

Treatment with forskolin leads to reduced proliferation that is dependent on PP2A activation

To assess whether increased PP2A activity affects cell proliferation of AML cells, KG-1 and HEL cell lines were treated with the PP2A activator forskolin or vehicle (DMSO). Phosphatase assays to quantify PP2A activity levels confirmed that forskolin treatment activates PP2A: forskolin induced a 1.5- to 2-fold increase in PP2A activity (Figure 2A). To study if a higher PP2A activity was associated with activation of PP2A protein, we pretreated KG-1 and HEL cells with the PP2A inhibitor okadaic acid for 2 hours, followed by incubation with forskolin or vehicle for 48 hours. Forskolin-induced PP2A activity in KG-1 and HEL cells was inhibited by okadaic acid (Figure 2A). Western blot analysis showed that similar levels of PP2Ac protein were immunoprecipitated in the PP2A phosphatase assays (figure 2B), suggesting that forskolin-induced PP2A activity is not due to changes in PP2Ac expression levels.

We next analyzed the effect of PP2A activation on cell growth using MTS assay. We observed a decreased in the proliferation of forskolin-treated KG-1 cells compared with vehicle-treated (Figure 2C). In addition, total cell counts and cell viability were confirmed with the Trypan Blue method (data not shown). These results show that PP2A activation by forskolin treatment induces toxicity in KG-1 cells. Similar results were obtained with the HEL cell line (Figure 2C). In addition, we observed that the impaired proliferation induced by forskolin was partially rescued by the treatment with the phosphatase inhibitor okadaic acid used at a concentration that inhibits PP2A but no other phosphatases.[44]

To confirm that the mechanism was mainly dependent on PP2A activation we performed the same experiments in HEL and KG-1 cell lines using the PP2A

activator FTY720 observing a similar effect with this drug than with forskolin (Supplementary Figure 1). Moreover, treatment with the PP2A activators forskolin and FTY720 had less effect in MUTZ-3 and Kasumi-1, the cell lines with PP2A low expression, than in HEL and KG-1, suggesting that the effect of those drugs in AML cells is mainly via PP2A activation (Supplementary Figure 2).

Increased PP2A activity by forskolin induces apoptosis in AML cells

To further investigate the biologic effect of the forskolin-induced PP2A activation in AML, KG-1 cells were treated with forskolin for 48 hours, and we assessed apoptosis with the caspase Glo-3/7 assay kit. Vehicle-treated cells were used as controls. Consistent with its ability to enhance PP2A activity and suppress cell proliferation, forskolin had a caspase-dependent proapoptotic effect, increasing caspase activity 6.5-fold in forskolin-treated KG-1 cells compared with vehicle-treated cells (Figure 3A). In addition, caspase activity in forskolin-treated cells was markedly reduced when cells were pretreated with okadaic acid or the caspase inhibitor Z-VAD-fmk. Effect in apoptosis was confirmed by an Annexin-V-based assay (Figure 3B).

Forskolin induces changes in the phosphorylation status of PP2A targets

We next analyzed by western blot the effects of the forskolin-induced PP2A activation at protein level. As expected, we observed that phosphorylation on tyrosine 307 of PP2Ac was negatively affected in cells treated with forskolin compared with cells treated with vehicle (DMSO) (Figure 3C). These data confirmed the results obtained with the PP2A phosphatase assays (Figure 2A). In addition, PP2Ac phosphorylation was restored when cells were pretreated with the PP2A inhibitor okadaic acid. Consistent with previous reports about the effects of PP2A activation in myeloid BCR-ABL positive cells,[31] forskolin treatment in AML BCR-ABL negative cells, decreased phosphorylation (activity) of the PP2A targets AKT and ERK1/2 without affecting their expression levels. Moreover, treatment with okadaic acid rescued AKT and ERK1/2 phosphorylation in forskolin-treated KG-1 cells (Figure 3C).

Additive effect of PP2A activation with Idarubicin and Ara-c treatments in AML cells

In order to assess the effect of a combination between standard induction chemotherapy drugs in AML and a PP2A activator, we treated KG-1 cells with either Idarubicin or Ara-c, alone or in combination with forskolin. Of importance, we observed that PP2A activation enhanced the antileukemic effects mediated by both Idarubicin (Figure 4A) and Ara-c (Figure 4B) treatments in KG-1 and Hel cell lines. Moreover, Chou-Talalay analyses showed that PP2A activation has an additive antitumoral effect when combined with either Idarubicin or Ara-c.

PP2A inhibition is a recurrent event in AML

To further evaluate the importance of PP2A in AML, we analyzed at protein level the prevalence of PP2A inhibition in a series of 37 patients with AML at

diagnosis. Patient characteristics are presented in Table 1. Increased phosphorylation of tyrosine 307 was observed in 29 out of 37 cases (78.4%) (Figure 5A and Supplementary Figure 3). In addition, PP2A activity was compared between samples of 8 AML patients and 3 normal controls, and we observed a significant reduction of PP2A activity in all the 8 patient samples analyzed (Supplementary Figure 4A). These results would indicate that PP2A inhibition is a recurrent event in AML. Moreover, we analyzed samples of 3 patients at diagnosis, complete remission and relapse, observing that PP2A phosphorylation decreases at complete remission, and increases at relapse (Figure 5B and Supplementary Figure 4B).

In order to investigate the possible causes of PP2A phosphorylation, we analyzed the expression of *SET*, *SETBP1* and *CIP2A*, and the presence of the constitutive activating mutation *JAK2*-V617F in this series of patients. We found overexpression of *SET* and/or *SETBP1* in 55% cases with increased PP2A phosphorylation (16/29), and in none of the cases with low phosphorylated levels (Table 2). *CIP2A* overexpression was detected only in two cases with high phosphorylation. Analysis by western blot confirmed CIP2A and SET overexpression also at the protein level (Supplementary Figure 5). In addition, *JAK2*-V617F was detected in one of the patients with high PP2A phosphorylation, although also in 3 cases with low PP2A phosphorylation (Supplementary Table 2). As detected in the cell lines, 6 out of 8 patient samples with low PP2A phosphorylation had a reduced expression of PP2A-C (Figure 5A). Interestingly, two patients had both PP2A inhibition and *FLT3* constitutively activated by the *FLT3*-ITD mutation (Supplementary Table 2), two aberrations that led to the activation of the transduction pathways JAK/STAT, ERK and AKT.[45]

Genome-wide gene expression analysis of PP2A subunits in myeloid cell lines

The fact that 12 of the patients included in our series had no known mechanism that could explain PP2A inactivation prompted us to perform expression arrays of 16 myeloid cell lines in order to obtain an overview of the expression of the PP2A subunits. One of these cases had a deletion del(11)(q13q23), a region where the locus of the PP2A scaffold subunit PP2A-Aβ (*PPP2R1B*) (11q23) and the regulatory subunit *PPP2R5B* (11q13) are located. We hypothesized that as previously described,[9] downregulation of some scaffold and regulatory subunits could affect the activity of PP2A.

Expression arrays of the cell lines showed a significant downregulation of the regulatory subunits *PPP2R5B* and *PPP2R5C* in both AML and BC-CML cell lines when compared with normal controls; interestingly, *PPP2R5B* was significantly more downregulated in AML than in CML (Supplementary Figure 6). Data were validated by QRT-PCR (Supplementary Table 1). Moreover, analysis of the patient samples revealed that PP2A-Aβ (*PPP2R1B*), *PPP2R5B* or/and *PPP2R5C* were downregulated in several AML patient samples (Supplementary Table 3). We could analyze at protein level 16 cases that had PPP2R5B downregulation by real time RT-PCR (QRT-PCR) and 15 out of 16 had decreased PPP2R5B protein expression. Moreover, 3 cases with no *PPP2R5B*

downregulation (P17-19) were included in the study and had normal PPP2R5B protein levels (Supplementary Figure 7A). We could not perform the study of PPP2R5C at protein level. With regard to PPP2R1B, there was no good correlation between mRNA and protein: only 8 out of the 17 cases analyzed had decreased PPP2R1B protein levels, the other 9 were normal. Moreover, we included in the study 5 cases with no *PPP2R1B* downregulation by QRT-PCR, and 4 had normal PPP2R1B protein levels (P6, P13, P17, P19) and one case had PPP2R1B low (Supplementary Figure 7B). Taken together, these results show that downregulation of these subunits is a common event in AML that could contribute to PP2A inactivation.

Discussion

PP2A is a human tumor suppressor that inhibits cellular transformation by regulating the activity of several signaling proteins critical for malignant cell behavior. We report here that PP2A inhibition is a recurrent event that could play an important role in AML. We demonstrate that PP2A activation by forskolin induce growth inhibition, caspase-dependent apoptosis, and the modification of downstream targets such as AKT and ERK1/2. Of importance, our data provides evidence that PP2A activation could be a promising therapeutic target in combination with drugs used in standard induction therapy, such as Idarubicin and Ara-c.

It has been reported that impaired PP2A activity plays a key role in BCR-ABL positive leukemias such as CML and ALL,[31-32] in myeloid precursors expressing imatinib-sensitive (V560G) and imatinib resistant (D816V) mutant c-KIT,[36] and also in CLL;[46] moreover, activation of PP2A by either FTY720 or forskolin seems to have promising therapeutic effects in these diseases. Many kinases have been reported to be deregulated in AML; however, the role of phosphatases in the cellular transformation of this disease remains underexplored.[10] As indicated above, only few studies have reported reduced PP2A activity in AML.[35-36] Our group has recently reported an impaired PP2A activity via SET as a consequence of SETBP1 overexpression, a recurrent event in AML (27%).[37] This leads us to hypothesize that PP2A inhibition could be a recurrent event in AML. Our results in both cell lines and patient samples confirm this hypothesis and show that PP2A inhibition plays an important role in AML transformation, since the pharmacologic activation of PP2A in vitro reverse some of the leukemogenic features (Figures 2 and 3; Supplementary Figures 1 and 2). When we investigated the PP2A status at protein level in 37 patients with AML at diagnosis, we observed PP2A inhibition in 78% of cases. Interestingly, we detected reduced PP2Ac expression in 6 out of 8 patient samples with no PP2A phosphorylation; this observation is consistent with the reduced PP2Ac levels observed in Kasumi-1 and MUTZ-3, and could suggest that a reduced protein expression could be a mechanism to decrease PP2A activity in AML. Furthermore, we also observed an additive effect of PP2A activation by forskolin with the antileukemic effects induced by Idarubicin or Ara-c in both KG-1 and HEL cells, suggesting that PP2A activation could be a new alternative for treating AML in combination with standard induction chemotherapy (Figure 4). It has been reported that forskolin-induced effects on cell growth and apoptosis of AML cells at the concentrations used in this study do not impair viability of normal bone marrow cells;[31] this observation would support the use of PP2A activators in future therapies for patients with AML. Moreover, we show that forskolin suppresses Akt and ERK1/2 function in an okadaic acid sensitive manner, indicating that its action in AML is dependent on PP2A activation (Figure 3). These results are also interesting since several PP2A targets have been reported to be deregulated in AML and some of them, as AKT, have been associated to poor outcome.[35] Our results support the premise that PP2A inactivation might be one of the events that contribute to these alterations, since PP2A plays an integral role in the regulation

of a number of major signaling pathways whose deregulation can contribute to cancer.[9]

Although evidence suggested that PP2A might be a tumor suppressor protein, recent findings provide convincing evidence that suppression of PP2A activity cooperates with other oncogenic changes to cause transformation of multiple cell types.[3,23,47] In our series we found that 34% of patients with PP2A inactivated (10/29) had either *FLT3* and/or *NPM1* mutated (Supplementary Table 2). Interestingly, the constitutive activation of *FLT3* in cases with *FLT3*-ITD also activates both Akt and ERK1/2,[48] suggesting that in some patients PP2A inactivation and *FLT3*-ITD could cooperate in the transformation of AML.

PP2A can be inhibited by the small tumor antigen of DNA tumor viruses, by upregulation of endogenous PP2A inhibitors, through mutational inactivation of the structural subunits, or by decreased expression of either the scaffold or the regulatory subunits.[9-10] On the other hand, JAK2 constitutive activity,[49-50] and *SETBP1* overexpression in BCR-ABL negative AML[37] might independently contribute to PP2A inactivation. Our results show that the mechanisms of PP2A inactivation in AML might be the overexpression of the physiological PP2A inhibitors *CIP2A*[33] and *SET*,[51] the overexpression of *SETBP1*, or the downregulation of PP2A subunits, suggesting that dysfunction of several distinct PP2A complexes may contribute to cell transformation. Overexpression of *CIP2A*, *SET* or *SETBP1* could explain the mechanism of PP2A inactivation in 58% of our cases (17/29). *SET* is upregulated in multiple solid tumors,[52] and has been reported to be fused to NUP214/CAN in a patient with AML.[53] Importantly, Neviani et al. (2005) demonstrated that PP2A inactivation in CML-BC results from increased expression of SET, which is induced by BCR-ABL in a dose- and kinase-dependent manner and, that like BCR-ABL, SET progressively increases during transition to blast crisis. In fact, imatinib treatment and SET downregulation restored PP2A activity back to normal levels.[31] Our results show that high expression of SET also leads to PP2A inactivation in AML, independently of BCR-ABL induction. In addition, the activating mutation *JAK2*-V617F was detected in one of the samples analyzed; however, 3 cases with *JAK2*-V617F had no PP2A hyperphosphorylation, suggesting that either this mutation would need other additional changes to inactivate PP2A or that *JAK2*-V617F is not causal in activating PP2A in AML.

Since in 12 cases the mechanism remained undetermined, and one case had a deletion del(11)(q13q23), a region where the loci of the PP2A scaffold subunit PP2A-Aβ (*PPP2R1B*) (11q23) and the regulatory subunit *PPP2R5B* (11q13) are located, we hypothesized that downregulation of some subunits could affect the activity of PP2A, as previously reported.[9] Analysis of 16 myeloid cell lines and AML patient samples showed genetic aberrations affecting PP2A subunits that could be playing an important role in the PP2A inhibition observed in AML. We found downregulation of *PPP2R5B* and *PPP2R5C* in both AML and CB-CML cell lines (Supplementary Figure 6). It has been reported that PPP2R5B is a tumor suppressor that negatively regulates Pim-1 protein kinase, which is known to enhance the ability of c-Myc to induce lymphomas.[26] Furthermore, it has been described that suppression of *PPP2R5C* expression contributes to the experimental transformation of human cells.[28] Our data suggest that loss of *PPP2R5B* and *PPP2R5C* could be playing a role in AML development

contributing to deregulate the correct PP2A function. We also found that downregulation of the Aβ subunit is a common event in AML. Most cellular PP2A holoenzymes contain the Aα isoform of the scaffold subunit, but a small fraction (10%) contain a second isoform termed Aβ. Although mutations that disrupt the ability of Aβ to form holoenzymes in vitro were identified in several types of cancer,[12,54-55] the report by Sablina et al.[21] provides the first hard evidence that loss of functional Aβ due to these cancer-associated mutations contributes to transformation.[3] Further studies are necessary to clarify the importance of the downregulation of these PP2A subunits in AML.

Taking together, our results suggest that functional inactivation of the PP2A tumor suppressor is a recurrent event that seems to represent an important mechanism in the leukemogenic transformation of AML. We show that functional loss of PP2A activity could occur through different contributing mechanisms such as enhancement of endogenous PP2A inhibitors, decreased PP2A expression of the structural or regulatory subunits of PP2A. Moreover, although PP2A activators are not still clinically available, the results obtained in this study suggest that PP2A activation could be considered as a future therapeutic alternative for AML. The knowledge that pharmacologic restoration of PP2A activity is able to antagonize leukemogenesis and has an additive effect with other drugs used in the treatment of AML highlights PP2A as a potential target for future therapies combined with PP2A activators.

Acknowledgements: This work was supported by the Ministerio Educación y Ciencia (SAF2005/06425 and AP2006-03038), Ministerio Ciencia e Innovación (PI081687), Departamento Salud del Gobierno de Navarra (14/2008), ISCIII-RTICC (RD06/0020/0078), and Fundación para la Investigación Médica Aplicada y UTE (Spain). We thank Dr. Anna Sablina for useful discussion, Elizabeth Guruceaga, of the Unit of Proteomics, Genomics and Bioinformatics of CIMA, for bioinformatics analysis, and Leyre Urquiza and Marisol Gonzalez-Huarriz for technical assistance.

Conflict of interest: Authors declare there are no competing financial interests in relation to the work described.

Tables

Table 1 Clinical and molecular characteristics of a series of 37 patients with AML.

		No. (%)
Sex		
	Male	22 (59.5)
	Female	15 (40.5)
Age		
	≤60 years	12 (33.3)
	>60 years	24 (66.7)
Complete remission		
	No	9 (32.2)
	Yes	19 (67.8)
Diagnosis		
	AML-M0	3 (8.2)
	AML-M1	7 (18.8)
	AML-M2	5 (13.6)
	AML-M4	9 (24.3)
	AML-M5	9 (24.3)
	AML-M6	2 (5.4)
	AML-M7	2 (5.4)
sAML		
	No	31 (83.8)
	Yes	6 (16.2)
Cytogenetic group		
	good	2 (5.4)
	intermediate	29 (78.4)
	poor	6 (16.2)
SETBP1 overexpression		
	No	25 (67.6)
	Yes	12 (32.4)
SET overexpression		
	No	26 (70.3)
	Yes	11 (29.7)
JAK2 (V617F)		
	No	31 (88.6)
	Yes	4 (11.4)
FLT3-ITD		
	No	29 (82.8)
	Yes	6 (17.2)
NPM1 mutated		
	No	18 (62.2)
	Yes	11 (37.8)
WT1 overexpression		
	No	8 (23.6)
	Yes	26 (76.4)
CIP2A overexpression		
	No	27 (90)
	Yes	3 (10)

Table 2 Overexpression of *SET* and *SETBP1*, and presence of *JAK2*-V617F in a series of 37 patients with AML.

	PP2A phosphorylated	PP2A no phosphorylated	Total
SET overexpression	4	0	4
SETBP1 overexpression	5	0	5
SET and *SETBP1* overexpression	7	0	7
JAK2-V617F	1	3	4
CIP2A overexpression	2	1	3
TOTAL	29	8	37

Figures

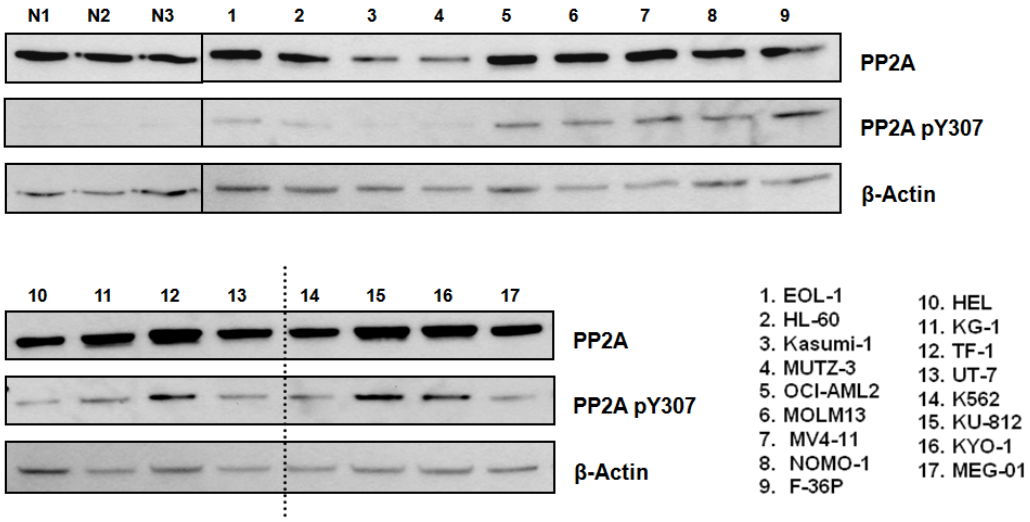


Figure 1 Analysis of PP2A activation and expression in 17 myeloid cell lines. Comparison of the PP2A-C expression and phosphorylation levels on tyrosine 307 by western blot in normal donors and in myeloid cell lines. N1-3: normal donors.

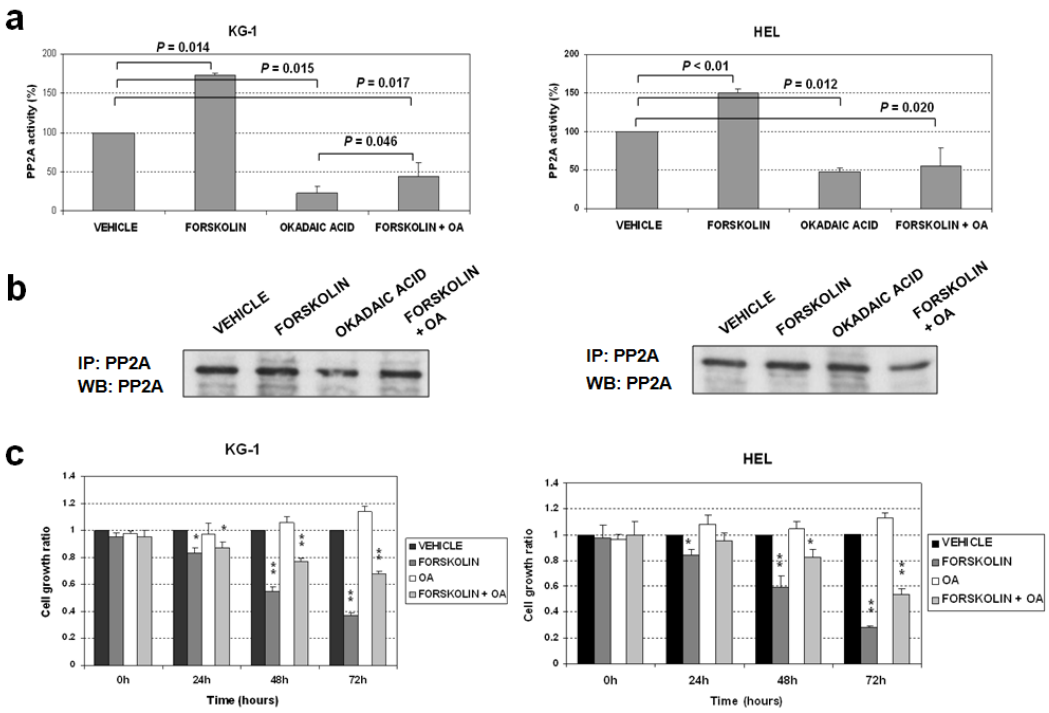


Figure 2 Forskolin treatment induces an inhibition of the proliferation that is dependent on PP2A activation. Forskolin was used at 40μM and okadaic acid at 2.5nM. Data represented are mean of three independent experiments ± s.d. (**a**) Forskolin-induced PP2A activity in KG-1 and HEL cell lines is inhibited by okadaic acid treatment. (**b**) Western blot analysis showing the levels of immunoprecipitated PP2A from the KG-1 and HEL lysates used in the phosphatase assays. (**c**) Inhibited proliferation induced by forskolin treatment is partially rescued by okadaic acid; * $P < 0.05$; ** $P < 0.01$.

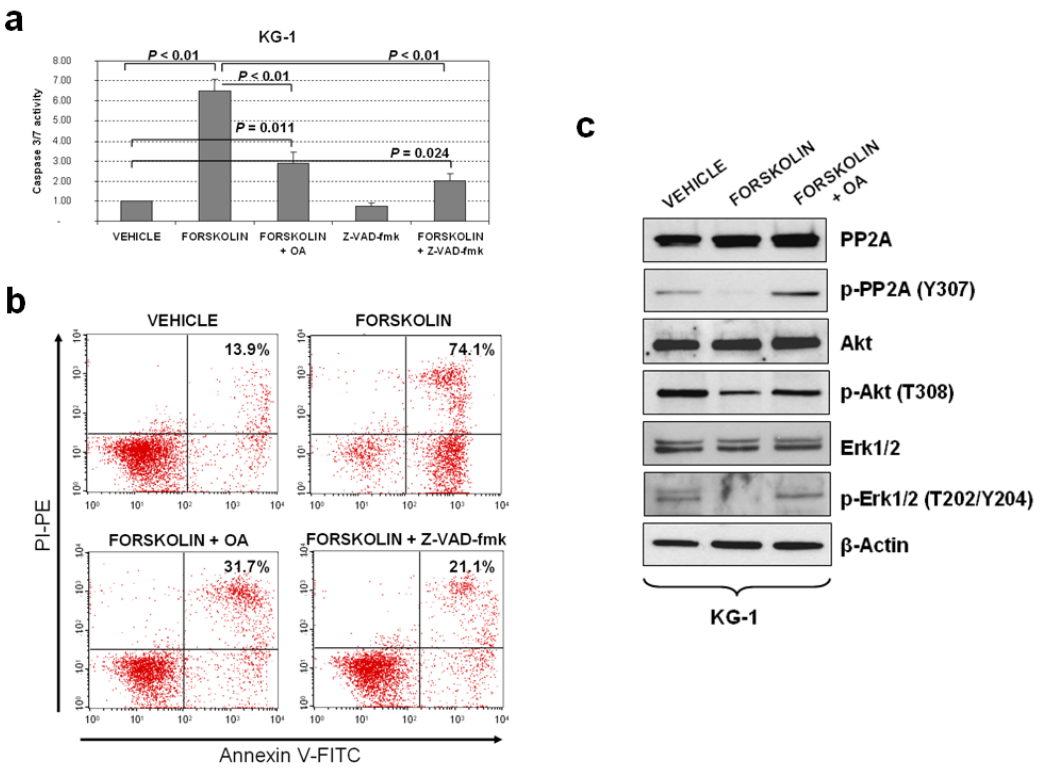


Figure 3 Forskolin induces caspase-dependent apoptosis together with changes in the phosphorylation status of PP2A targets. Forskolin was used at 40μM and okadaic acid at 2.5nM. Z-VAD-fmk was used at 150mM. Data represented are mean of three independent experiments ± s.d. (**a**) Caspase 3/7 assays in untreated, forskolin-treated, forskolin/okadaic acid-treated, Z-VAD-fmk-treated and forskolin/Z-VAD-fmk-treated KG-1 cells. (**b**) Annexin-V/propidium iodide assays in untreated, forskolin-treated, forskolin/okadaic acid-treated and forskolin/Z-VAD-fmk-treated KG-1 cells. (**c**) Western blot showing the effect of forskolin and forskolin/okadaic acid treatments in KG-1 cells on PP2A, AKT and ERK1/2 activity and expression.

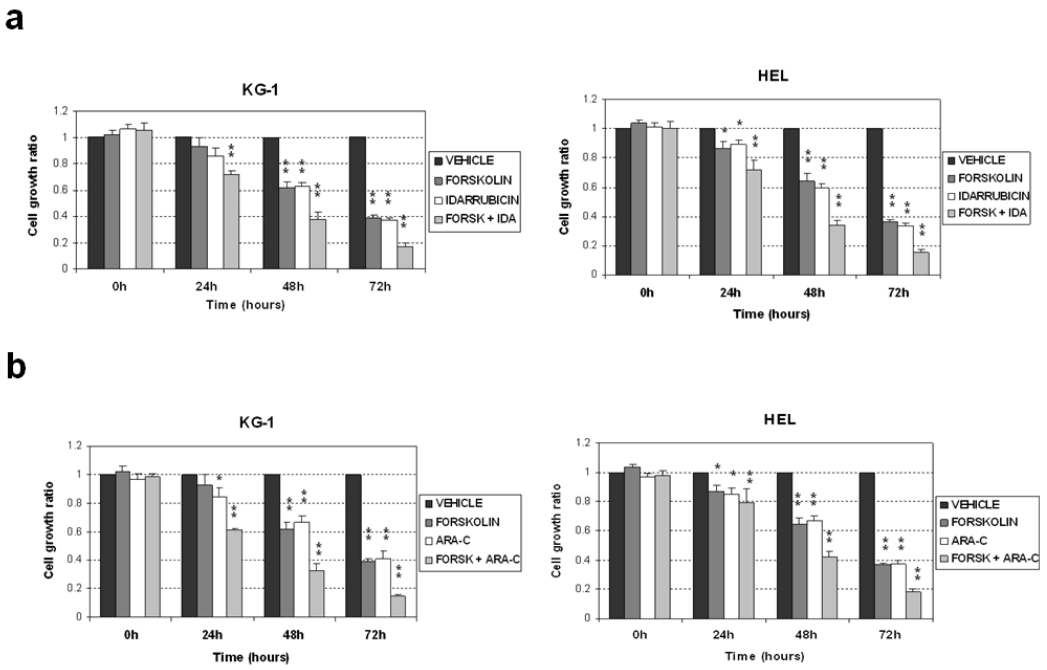


Figure 4 PP2A activation boosts antileukemic effects of Idarubicin and Ara-c treatments. Data represented are mean of three independent experiments ± s.d.; * $P < 0.05$; ** $P < 0.01$; (**a**) MTS assays showing the effect of PP2A activation by forskolin (40μM) on the cell growth ratio of KG-1 and HEL cells treated Idarubicin (15nM). (**b**) MTS assays showing the effect of PP2A activation by forskolin (40 μM) on the cell growth ratio of KG-1 and HEL cells treated Ara-c (2.5μM). Cells treated with vehicle (DMSO) were used as controls.

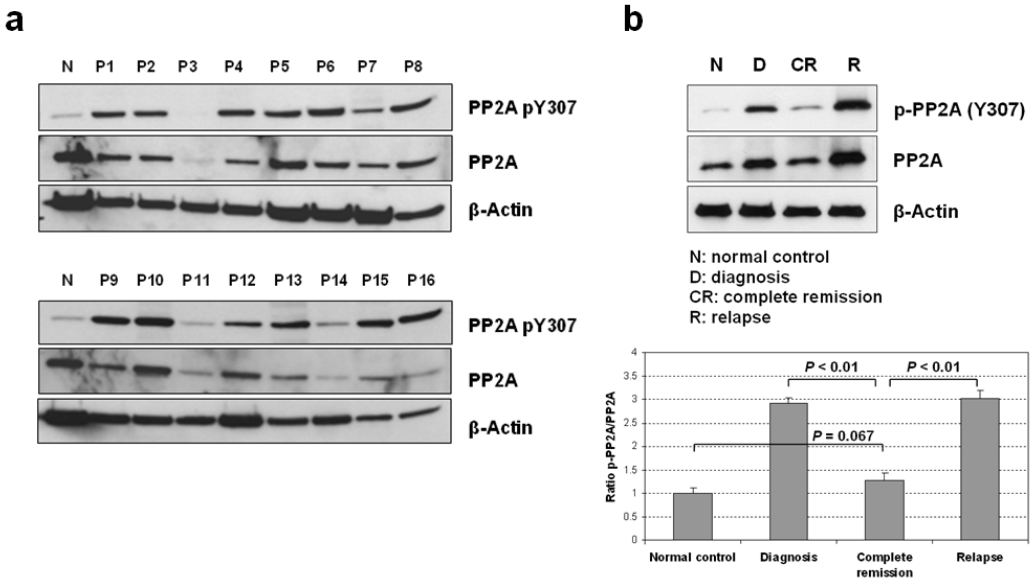


Figure 5 Comparison by western-blot of the PP2Ac expression and activity levels between patient samples and normal donors. (**a**) Analysis of PP2A in 16 samples of AML patients at diagnosis. (**b**) Comparison of PP2A activation in samples of a patient with AML at diagnosis, complete remission and relapse, including a densitometric analysis of the p-PP2A/PP2A ratio.

References

1 Rowe JM. Optimal induction and post-remission therapy for AML in first remission. *Hematology Am Soc Hematol Educ Program* 2009: 396-405.

2 Haferlach T. Molecular genetic pathways as therapeutic targets in acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program* 2008: 400-411.

3 Mumby M. PP2A: unveiling a reluctant tumor suppressor. *Cell* 2007; **130**: 21-24.

4 Millward TA, Zolnierowicz S,Hemmings BA. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem Sci* 1999; **24**: 186-191.

5 Hemmings BA, Adams-Pearson C, Maurer F, Muller P, Goris J, Merlevede W *et al.* alpha- and beta-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry* 1990; **29**: 3166-3173.

6 Zhou J, Pham HT, Ruediger R,Walter G. Characterization of the Aalpha and Abeta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. *Biochem J* 2003; **369**: 387-398.

7 Arino J, Woon CW, Brautigan DL, Miller TB, Jr.,Johnson GL. Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. *Proc Natl Acad Sci U S A* 1988; **85**: 4252-4256.

8 Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem* 1989; **58**: 453-508.

9 Eichhorn PJ, Creyghton MP,Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta* 2009; **1795**: 1-15.

10 Westermarck J,Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* 2008; **14**: 152-160.

11 Ruediger R, Pham HT,Walter G. Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. *Oncogene* 2001; **20**: 10-15.

12 Ruediger R, Pham HT,Walter G. Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. *Oncogene* 2001; **20**: 1892-1899.

13 Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J *et al.* Alterations of the PPP2R1B gene in human lung and colon cancer. *Science* 1998; **282**: 284-287.

14 Calin GA, di Iasio MG, Caprini E, Vorechovsky I, Natali PG, Sozzi G *et al.* Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms. *Oncogene* 2000; **19**: 1191-1195.

15 Esplin ED, Ramos P, Martinez B, Tomlinson GE, Mumby MC,Evans GA. The glycine 90 to aspartate alteration in the Abeta subunit of PP2A

(PPP2R1B) associates with breast cancer and causes a deficit in protein function. *Genes Chromosomes Cancer* 2006; **45**: 182-190.

16 Takagi Y, Futamura M, Yamaguchi K, Aoki S, Takahashi T,Saji S. Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. *Gut* 2000; **47**: 268-271.

17 Tamaki M, Goi T, Hirono Y, Katayama K,Yamaguchi A. PPP2R1B gene alterations inhibit interaction of PP2A-Abeta and PP2A-C proteins in colorectal cancers. *Oncol Rep* 2004; **11**: 655-659.

18 Kalla C, Scheuermann MO, Kube I, Schlotter M, Mertens D, Dohner H *et al.* Analysis of 11q22-q23 deletion target genes in B-cell chronic lymphocytic leukaemia: evidence for a pathogenic role of NPAT, CUL5, and PPP2R1B. *Eur J Cancer* 2007; **43**: 1328-1335.

19 Hemmer S, Wasenius VM, Haglund C, Zhu Y, Knuutila S, Franssila K *et al.* Alterations in the suppressor gene PPP2R1B in parathyroid hyperplasias and adenomas. *Cancer Genet Cytogenet* 2002; **134**: 13-17.

20 Chou HC, Chen CH, Lee HS, Lee CZ, Huang GT, Yang PM *et al.* Alterations of tumour suppressor gene PPP2R1B in hepatocellular carcinoma. *Cancer Lett* 2007; **253**: 138-143.

21 Sablina AA, Chen W, Arroyo JD, Corral L, Hector M, Bulmer SE *et al.* The tumor suppressor PP2A Abeta regulates the RalA GTPase. *Cell* 2007; **129**: 969-982.

22 Chen W, Arroyo JD, Timmons JC, Possemato R,Hahn WC. Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. *Cancer Res* 2005; **65**: 8183-8192.

23 Sablina AA,Hahn WC. The role of PP2A A subunits in tumor suppression. *Cell Adh Migr* 2007; **1**: 140-141.

24 Arnold HK,Sears RC. Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. *Mol Cell Biol* 2006; **26**: 2832-2844.

25 Margolis SS, Perry JA, Forester CM, Nutt LK, Guo Y, Jardim MJ *et al.* Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell* 2006; **127**: 759-773.

26 Ma J, Arnold HK, Lilly MB, Sears RC,Kraft AS. Negative regulation of Pim-1 protein kinase levels by the B56beta subunit of PP2A. *Oncogene* 2007; **26**: 5145-5153.

27 Letourneux C, Rocher G,Porteu F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *EMBO J* 2006; **25**: 727-738.

28 Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC,Hahn WC. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* 2004; **5**: 127-136.

29 Shouse GP, Nobumori Y,Liu X. A B56gamma mutation in lung cancer disrupts the p53-dependent tumor-suppressor function of protein phosphatase 2A. *Oncogene* 2010.

30 Li HH, Cai X, Shouse GP, Piluso LG,Liu X. A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J* 2007; **26**: 402-411.

31 Neviani P, Santhanam R, Trotta R, Notari M, Blaser BW, Liu S *et al.* The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR-ABL-regulated SET protein. *Cancer Cell* 2005; **8**: 355-368.

32 Neviani P, Santhanam R, Oaks JJ, Eiring AM, Notari M, Blaser BW *et al.* FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest* 2007; **117**: 2408-2421.

33 Junttila MR, Puustinen P, Niemela M, Ahola R, Arnold H, Bottzauw T *et al.* CIP2A inhibits PP2A in human malignancies. *Cell* 2007; **130**: 51-62.

34 Yokoyama N, Reich NC,Miller WT. Determinants for the interaction between Janus kinase 2 and protein phosphatase 2A. *Arch Biochem Biophys* 2003; **417**: 87-95.

35 Gallay N, Dos Santos C, Cuzin L, Bousquet M, Simmonet Gouy V, Chaussade C *et al.* The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* 2009; **23**: 1029-1038.

36 Roberts KG, Smith AM, McDougall F, Carpenter H, Horan M, Neviani P *et al.* Essential Requirement for PP2A Inhibition by the Oncogenic Receptor c-KIT Suggests PP2A Reactivation as a Strategy to Treat c-KIT+ Cancers. *Cancer Res* 2010.

37 Cristobal I, Blanco FJ, Garcia-Orti L, Marcotegui N, Vicente C, Rifon J *et al.* SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood* 2010; **115**: 615-625.

38 Luo LY, Huang J, Gou BD, Zhang TL,Wang K. Induction of human promyelocytic leukemia HL-60 cell differentiation into monocytes by arsenic sulphide: involvement of serine/threonine protein phosphatases. *Leuk Res* 2006; **30**: 1399-1405.

39 Martelli AM, Papa V, Tazzari PL, Ricci F, Evangelisti C, Chiarini F *et al.* Erucylphosphohomocholine, the first intravenously applicable alkylphosphocholine, is cytotoxic to acute myelogenous leukemia cells through JNK- and PP2A-dependent mechanisms. *Leukemia* 2010; **24**: 687-698.

40 Livak KJ,Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408.

41 Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B,Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003; **31**: e15.

42 Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article3.

43 Chen J, Martin BL,Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science* 1992; **257**: 1261-1264.

44 Saydam G, Aydin HH, Sahin F, Selvi N, Oktem G, Terzioglu E *et al.* Involvement of protein phosphatase 2A in interferon-alpha-2b-induced apoptosis in K562 human chronic myelogenous leukaemia cells. *Leuk Res* 2003; **27**: 709-717.

45 Gilliland DG,Griffin JD. Role of FLT3 in leukemia. *Curr Opin Hematol* 2002; **9**: 274-281.

46 Liu Q, Zhao X, Frissora F, Ma Y, Santhanam R, Jarjoura D *et al.* FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. *Blood* 2008; **111**: 275-284.

47 Junttila MR,Westermarck J. Mechanisms of MYC stabilization in human malignancies. *Cell Cycle* 2008; **7**: 592-596.

48 Kornblau SM, Singh N, Qiu Y, Chen W, Zhang N,Coombes KR. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. *Clin Cancer Res* 2010; **16**: 1865-1874.

49 Samanta AK, Chakraborty SN, Wang Y, Kantarjian H, Sun X, Hood J *et al.* Jak2 inhibition deactivates Lyn kinase through the SET-PP2A-SHP1 pathway, causing apoptosis in drug-resistant cells from chronic myelogenous leukemia patients. *Oncogene* 2009; **28**: 1669-1681.

50 Yokoyama N, Reich NC,Miller WT. Involvement of protein phosphatase 2A in the interleukin-3-stimulated Jak2-Stat5 signaling pathway. *J Interferon Cytokine Res* 2001; **21**: 369-378.

51 Li M, Makkinje A,Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* 1996; **271**: 11059-11062.

52 Cervoni N, Detich N, Seo SB, Chakravarti D,Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. *J Biol Chem* 2002; **277**: 25026-25031.

53 Rosati R, La Starza R, Barba G, Gorello P, Pierini V, Matteucci C *et al.* Cryptic chromosome 9q34 deletion generates TAF-Ialpha/CAN and TAF-Ibeta/CAN fusion transcripts in acute myeloid leukemia. *Haematologica* 2007; **92**: 232-235.

54 Xu Y, Xing Y, Chen Y, Chao Y, Lin Z, Fan E *et al.* Structure of the protein phosphatase 2A holoenzyme. *Cell* 2006; **127**: 1239-1251.

55 Cho US, Xu W. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. *Nature* 2007; **445**: 53-57.

Supplementary Information

Supplementary Table 1 Quantification of the expression of PP2A-Cα (*PPP2CA*), P PP2A-Cβ (*PPP2CB*), PP2A-Aβ (*PPP2R1B*), *PPP2R5B* and *PPP2R5C* by real-time PCR in 17 myeloid cell lines. $2^{-\Delta\Delta C}{}_T$ represents normalized gene amount in a cell line relative to normal controls.

Cell line	$2^{-\Delta\Delta C}{}_T$ (*PPP2CA*)	$2^{-\Delta\Delta C}{}_T$ (*PPP2CB*)	$2^{-\Delta\Delta C}{}_T$ (*PPP2R1B*)	$2^{-\Delta\Delta C}{}_T$ (*PPP2R5B*)	$2^{-\Delta\Delta C}{}_T$ (*PPP2R5C*)
EOL-1	1.29	0.98	0.41	0.09	0.78
HL-60	0.82	0.48	0.23	0.05	0.63
Kasumi-1	1.18	0.48	0.28	0.05	0.26
MUTZ-3	1.39	0.43	0.50	0.06	0.46
OCI-AML2	0.72	0.55	0.34	0.08	0.54
MOLM13	1.03	0.77	0.22	0.03	0.39
MV4-11	2.19	0.82	0.38	0.08	0.91
NOMO-1	0.83	1.24	0.46	0.04	0.56
F-36P	3.84	3.61	0.27	0.13	0.49
HEL	1.01	1.21	0.34	0.07	0.43
KG-1	0.85	0.42	0.72	0.09	0.59
TF-1	2.17	1.01	0.77	0.10	1.01
UT-7	1.77	1.72	0.60	0.18	0.96
K562	1.80	2.51	0.59	0.28	0.74
KU-812	1.91	2.39	0.54	0.36	1.23
KYO-1	3.14	2.77	0.63	0.77	0.56
MEG-01	3.39	1.80	0.50	0.42	0.61

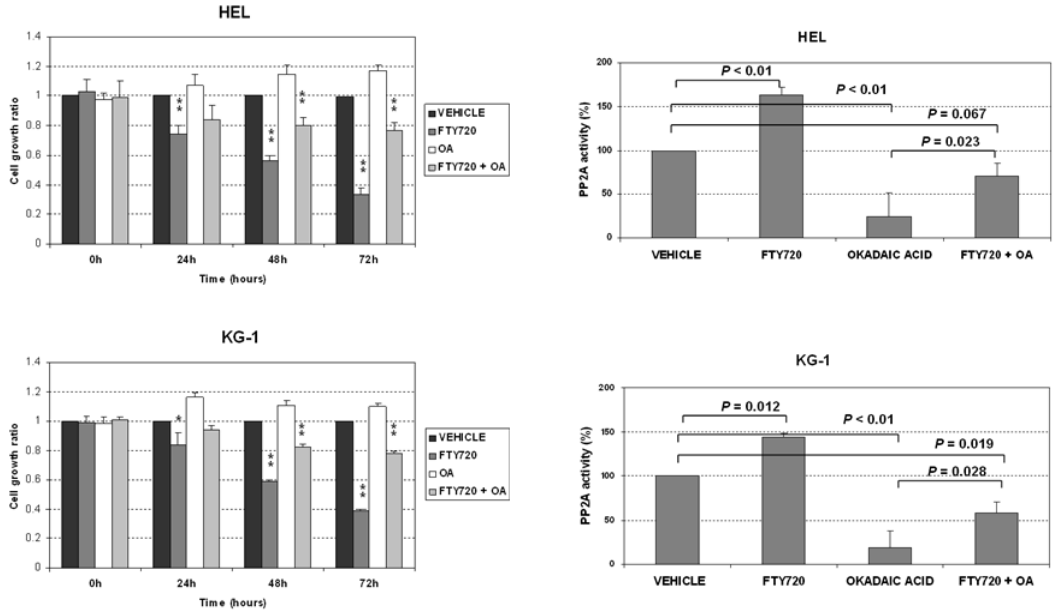
Cut-off (downregulation): *PPP2CA*: 0.63; *PPP2CB*: 0.60; *PPP2R1B*: 0.67; *PPP2R5B*: 0.39; *PPP2R5C*: 0.65

Supplementary Table 2 Clinical and molecular characteristics of the 37 patients with AML included in the study.

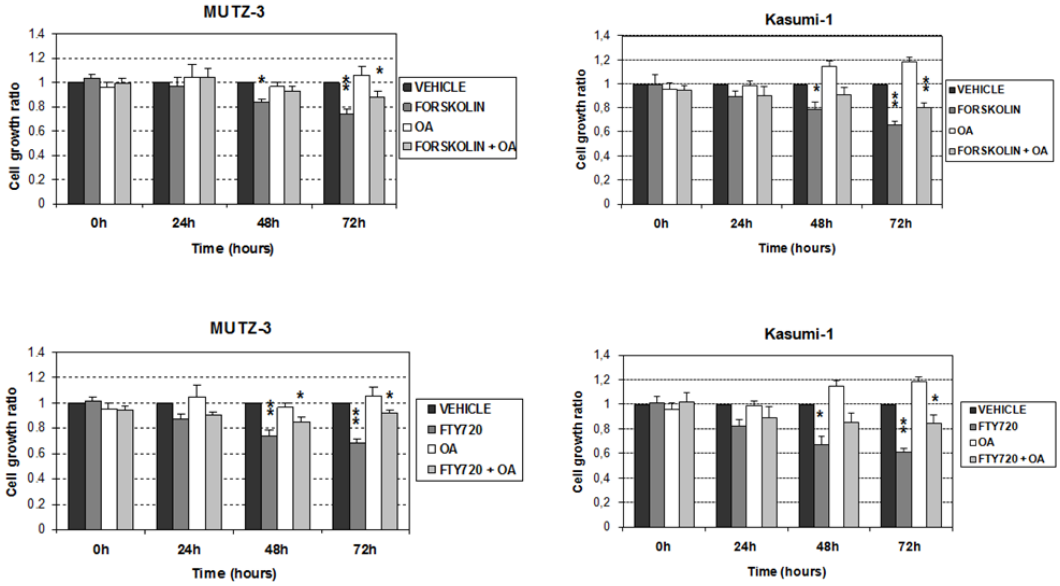
Case	Sex	Age	FAB classification	AML type	Karyotype	PP2A hyper-phosphorylation	PP2Ac protein expression	*SET* OE	*SETBP1* OE	*CIP2A* OE	*JAK2*-V617F	*PPP2R1B* DR	*PPP2R5B* DR	*PPP2R5C* DR	*FLT3*-ITD	*NPM1* mutated
17695	M	56	AML-M2	de novo	47,XY,+8	no	reduced	no	no	yes	yes	yes	no	yes	no	no
19608	F	76	AML-M2	sAML	46,XX	no	reduced	no	no	ND	yes	yes	yes	yes	no	no
24860	F	42	AML-M5	de novo	48,XX,del(1)(p21),+4,+6,del(11)(q21),der(11)add(11)(p15)	no	reduced	no	no	no	no	yes	ND	ND	no	no
22271	M	66	AML-M1	sAML	46,XY	no	reduced	no	no	no	yes	yes	yes	no	no	no
19820	F	46	AML-M4	de novo	46,XX	no	normal	no	no	ND	no	ND	ND	ND	yes	ND
21507	F	53	AML-M4	de novo	46,XX	no	reduced	no	no	no	no	yes	yes	yes	no	yes
25222	F	64	AML-M5	de novo	46,XX	no	reduced	no	no	no	no	yes	yes	yes	no	yes
26164	M	72	AML-M5	de novo	47,XY,der(3),+8	no	normal	no	no	no	no	ND	ND	ND	no	ND
22892	M	68	AML-M2	sAML	46,XY	yes	reduced	no	no	no	yes	yes	no	no	yes	no
15619	M	74	AML-M5	de novo	46,XY	yes	normal	yes	no	no	no	yes	yes	yes	no	ND
19937	M	43	AML-M5	de novo	46,XY	yes	normal	yes	no	ND	no	yes	no	yes	no	yes
24415	M	73	AML-M4	de novo	46,XY	yes	reduced	yes	no	no	no	yes	ND	no	no	yes
27578	M	71	AML-M1	de novo	46,XY	yes	normal	yes	no	no	no	yes	yes	yes	yes	yes
18154	M	ND	AML-M0	de novo	46,XY	yes	normal	no	yes	yes	no	no	no	no	no	no
19449	M	76	AML-M1	de novo	46,XY/47,XY,+13	yes	normal	no	yes	ND	no	no	no	ND	no	no
24212	F	59	AML-M5	de novo	46,XX	yes	reduced	no	yes	no	no	yes	no	yes	no	no
25448	F	69	AML-M7	de novo	46,XX,add(3)(q?)	yes	normal	no	yes	no	no	yes	yes	yes	no	no
32014	M	76	AML-M6	de novo	46,XY,t(12;18)(p13;q22)/47,XY,idem,+19	yes	normal	no	yes	no	ND	yes	yes	yes	ND	no
14412	F	61	AML-M4	de novo	46,XX	yes	normal	yes	yes	ND	no	no	ND	yes	yes	yes
18425	M	64	AML-M1	de novo	45,XY,-7,del(11)(q23),-14,-17,-21,+3mar/88,XXXX,cx	yes	normal	yes	yes	no	no	yes	no	no	no	no
25297	F	69	AML-M7	de novo	46,XX/46,XX,add(3)(q?)	yes	normal	yes	yes	no	ND	no	no	no	ND	ND
27101	M	40	AML-M4	de novo	ND	yes	normal	yes	yes	yes	no	no	no	no	no	no
27298	F	67	AML-M1	de novo	46,XX	yes	reduced	yes	yes	no	no	yes	no	no	yes	yes
27990	M	41	AML-M4	de novo	ND	yes	normal	yes	yes	no	no	no	no	no	no	no
28685	M	75	AML-M0	de novo	46,XY,t(11;12)(q13;p13),der(12),del(17)(p13),der(17)	yes	reduced	yes	yes	ND	no	yes	no	no	no	ND
17393	M	67	AML-M5	de novo	46,XY	yes	reduced	no	no	no	no	no	yes	yes	no	yes
17619	F	65	AML-M4	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	no	yes
18937	M	6	AML-M4	de novo	46,XY/46,XY,inv(7)(p21q36),del(11)(q13q23)	yes	reduced	no	no	ND	no	yes	yes	no	no	ND
20762	F	73	AML-M4	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	yes	yes
21720	F	55	AML-M0	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	no	no
23816	F	64	AML-M5	sAML	46,XX	yes	reduced	no	no	no	no	yes	yes	yes	no	yes
24043	M	71	AML-M1	sAML	46,XY,+8	yes	normal	no	no	no	no	yes	yes	yes	no	no
24731	M	55	AML-M1	de novo	46,XY	yes	normal	no	no	no	no	yes	yes	no	no	no
24756	F	59	AML-M2	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	no	no
26916	M	64	AML-M2	de novo	47,XY,+8	yes	normal	no	no	no	no	yes	yes	yes	no	ND
27535	M	71	AML-M6	sAML	47,XY,del(20)(q11)x2	yes	normal	no	no	no	no	yes	yes	yes	no	ND
28827	M	75	AML-M5	de novo	47,XY,+19	yes	reduced	no	no	no	no	yes	no	yes	no	no

Supplementary Table 3 Downregulation of PP2A-Aβ (*PPP2R1B*), *PPP2R5B* and *PPP2R5C* in a series of 37 patients with AML.

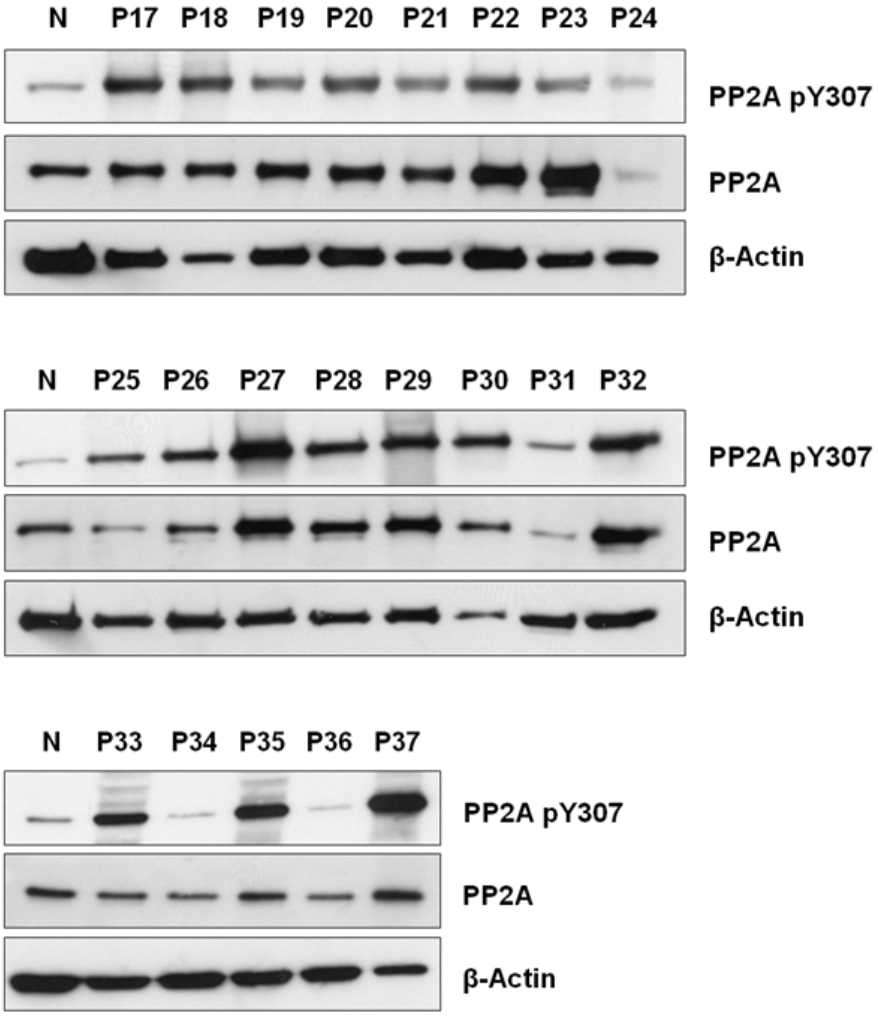
	Total number of cases (No. 37)	**PP2A phosphorylated**	**PP2A not phosphorylated**
PP2A-Aβ (PPP2R1B)			
No downregulation	7 (20%)	7 (24.2%)	0
Downregulation	28 (80%)	22 (75.8%)	6 (100%)
PPP2R5B			
No downregulation	13 (41.6%)	11 (42.3%)	2 (33.3%)
Downregulation	19 (59.4%)	15 (57.7%)	4 (66.7%)
PPP2R5C			
No downregulation	12 (36.4%)	11 (39.3%)	1 (20%)
Downregulation	21 (63.6%)	17 (60.7%)	4 (80%)



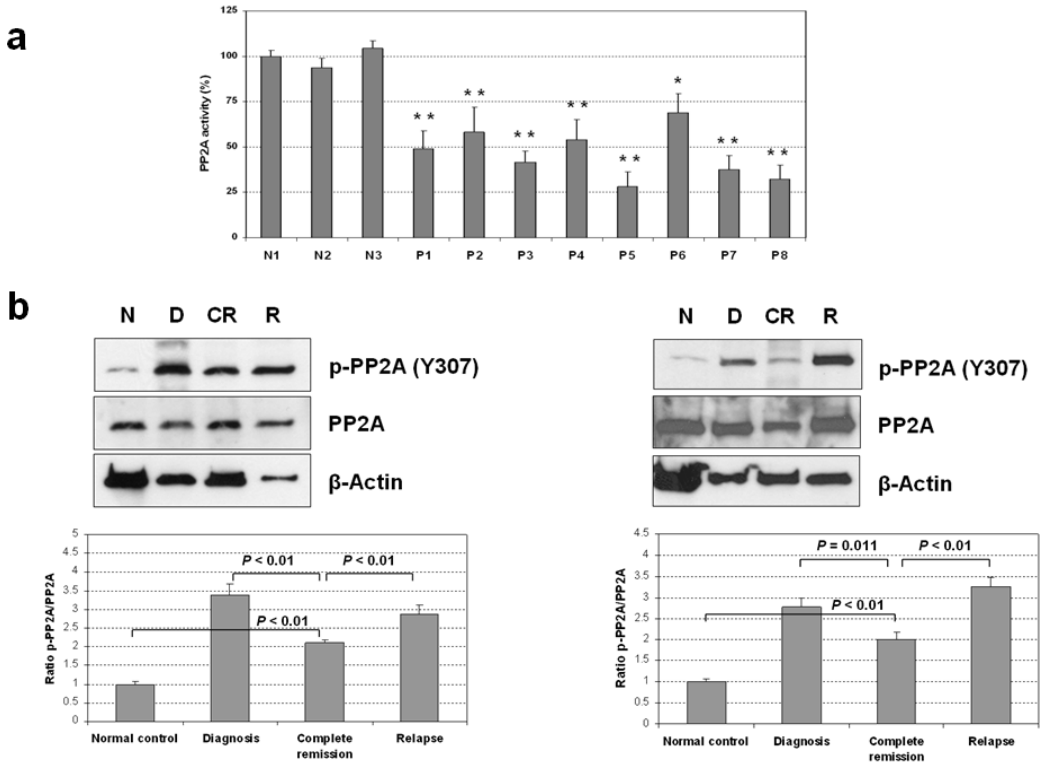
Supplementary Figure 1 FTY720-induced PP2A activity and impaired proliferation in KG-1 and HEL cell lines is inhibited by okadaic acid treatment. FTY720 was used at 10μM and okadaic acid at 2.5nM. Data represented are mean of three independent experiments ± s.d.; * $P < 0.05$; ** $P < 0.01$.



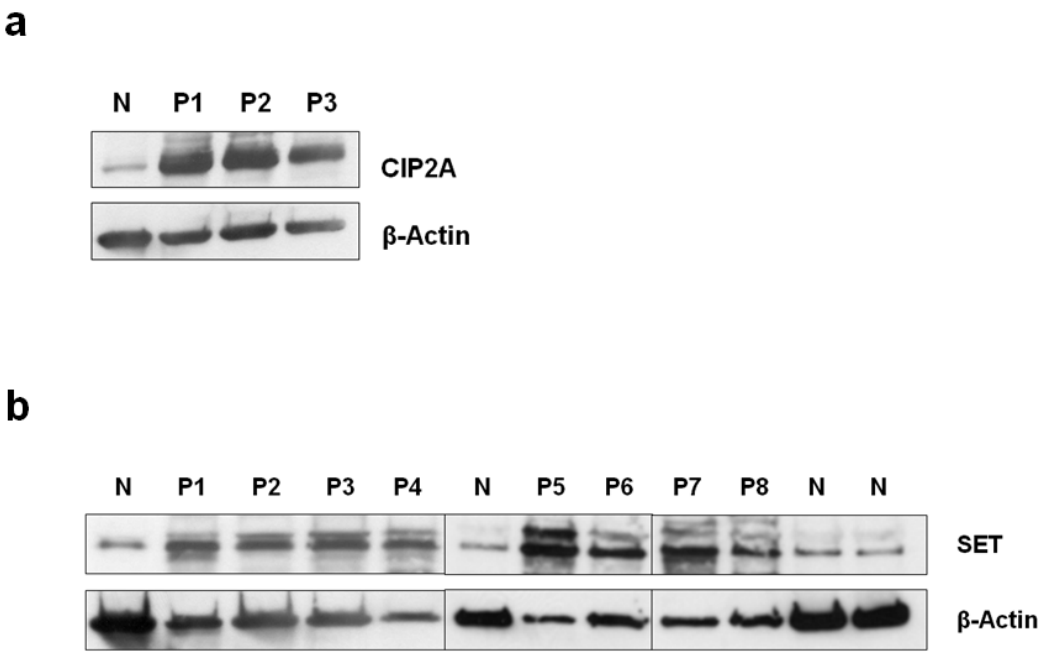
Supplementary Figure 2 Forskolin and FTY720 treatments show a lesser effect on the proliferation of the PP2A low cell lines MUTZ-3 and Kasumi-1. FTY720 was used at 10µM, forskolin at 40µM and okadaic acid at 2.5nM. Data represented are mean of three independent experiments ± s.d.; * $P < 0.05$; ** $P < 0.01$.



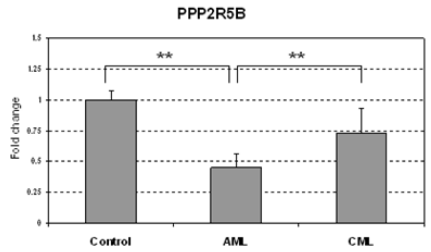
Supplementary Figure 3 Western blot analysis showing PP2A activation status in 21 AML patients samples at diagnosis.



Supplementary Figure 4 PP2A show reduced activity in AML patients. (**a**) PP2A activity in 8 AML patients at diagnosis compared to 3 normal controls; * $P < 0.05$; ** $P < 0.01$; N1-3: normal controls; P1-8: samples of AML patients at diagnosis (**b**) Comparison of PP2A activation in two AML patients with samples at diagnosis, complete remission and relapse, including densitometric analysis of p-PP2A/PP2A ratios; N: normal control; D: diagnosis; CR: complete remission; R: relapse.



Supplementary Figure 5 Western blot analysis of CIP2A (**a**) and SET (**b**) expression in AML patient samples at diagnosis; N: normal control; P: patient sample.

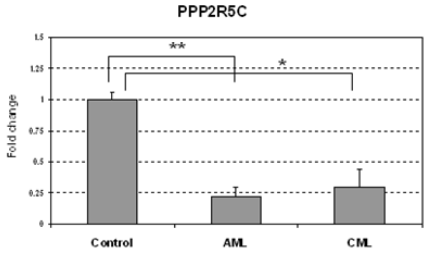


AML vs Control

Gene	Locus	Fold change	P-value	Adj. P-value	B
PPP2R5B	11q12-q13	0.451	<0.001	**0.001**	**1.534**
PPP2R5C	14q32	0.221	<0.001	**0.003**	**0.463**

CML vs Control

Gene	Locus	Fold change	P-value	Adj. P-value	B
PPP2R5B	11q12-q13	0.735	0.084	0.250	-5.557
PPP2R5C	14q32	0.296	0.002	**0.024**	-2.384

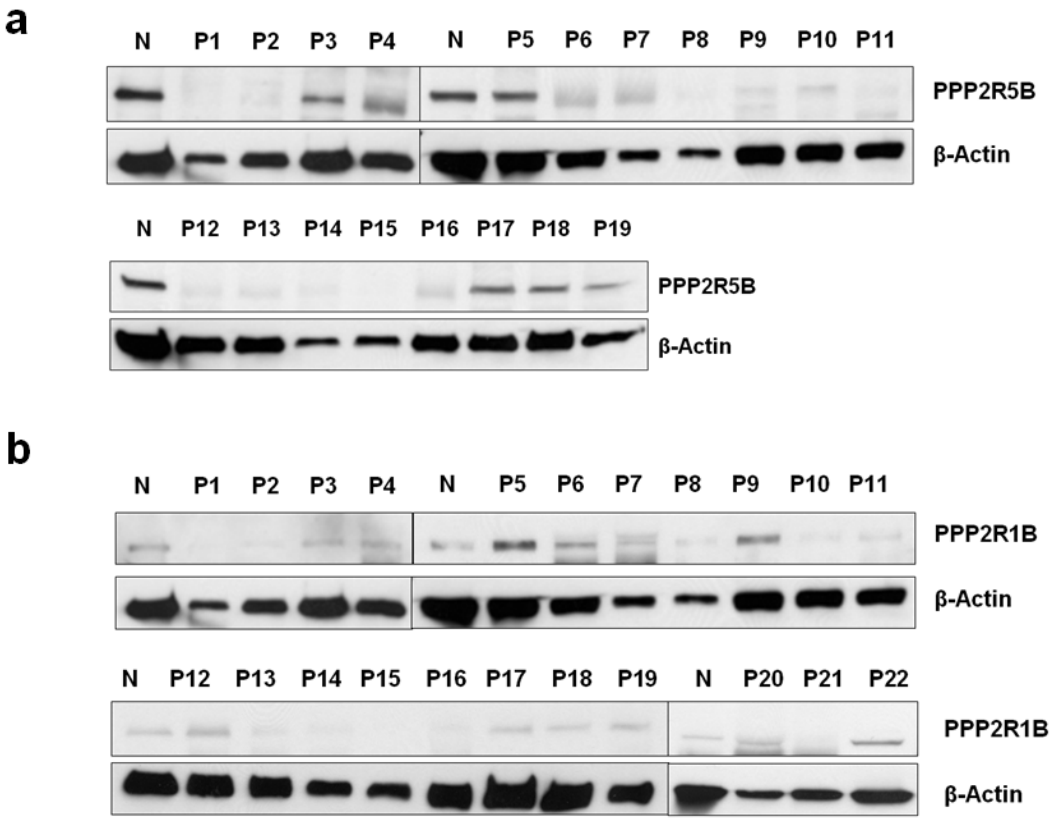


AML vs CML

Gene	Locus	Fold change	P-value	Adj. P-value	B
PPP2R5B	11q12-q13	0.618	<0.001	**0.019**	**1.342**
PPP2R5C	14q32	0.745	0.238	0.568	-5.493

*** Adj.P-value <0.05** **** B >0**

Supplementary Figure 6 Significant alterations affecting PP2A subunits observed from analysis of expression array data. Samples were distributed in three different groups: "Control" including 3 normal control samples, "AML" including 12 AML cell lines, and "CML" including 4 CML cell lines. Data represented are the gene expression mean of each group ± s.d.



Supplementary Figure 7 Western blot analysis of PPP2R5B (**a**) and PPP2R1B (**b**) expression in AML patient samples at diagnosis; N: normal control; P: patient sample.

<u>Capítulo 3.</u> *SETBP1* overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia

(artículo publicado en la revista *Blood*)

MYELOID NEOPLASIA

SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia

Ion Cristóbal,[1,2] Francisco J. Blanco,[3] Laura Garcia-Orti,[1] Nerea Marcotegui,[1] Carmen Vicente,[1,2] José Rifon,[4] Francisco J. Novo,[2] Eva Bandres,[1] María J. Calasanz,[2] Carmelo Bernabeu,[3] and María D. Odero[1,2]

[1]Division of Oncology, Center for Applied Medical Research, and [2]Department of Genetics, University of Navarra, Pamplona; [3]Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), and CIBER de Enfermedades Raras (CIBERER), Madrid; and [4]Clínica Universidad de Navarra, University of Navarra, Pamplona, Spain

SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia

Ion Cristóbal,[1,2] Francisco J. Blanco,[3] Laura Garcia-Orti,[1] Nerea Marcotegui,[1] Carmen Vicente,[1,2] José Rifon,[4] Francisco J. Novo,[2] Eva Bandres,[1] María J. Calasanz,[2] Carmelo Bernabeu,[3] María D. Odero[1,2]

[1]Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain;

[2]Department of Genetics, University of Navarra, Pamplona, Spain;

[3]Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), and CIBER de Enfermedades Raras (CIBERER), Madrid, Spain;

[4]Clínica Universidad de Navarra, University of Navarra, Pamplona, Spain

Keywords: *SETBP1*, overexpression, AML, prognosis

Running Title: *SETBP1* overexpression in AML

Scientific category: Myeloid Neoplasia

Corresponding author: María D. Odero, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pio XII-55 31008-Pamplona, Spain. E-mail: modero@unav.es. Phone: +34-948-194700. Fax: +34-948-194714.

Abstract

Acute myeloid leukemias (AML) result from multiple genetic alterations in hematopoietic stem cells. We describe a novel t(12;18)(p13;q12) involving *ETV6* in a patient with AML. The translocation resulted in overexpression of *SETBP1* (18q12), located close to the breakpoint. Overexpression of *SETBP1* through retroviral insertion has been reported to confer growth advantage in hematopoietic progenitor cells. We show that *SETBP1* overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein, and leading to the formation of a SETBP1-SET-PP2A complex that results in PP2A inhibition, promoting proliferation of the leukemic cells. The prevalence of *SETBP1* overexpression in AML at diagnosis (n=192) was 27.6%, and was associated with unfavorable cytogenetic prognostic group, monosomy 7, and *EVI1* overexpression ($p<0.01$). Patients with *SETBP1* overexpression had a significantly shorter overall survival (OS), and the prognosis impact was remarkably poor in patients older than 60 years in both OS ($p=0.015$) and event free survival ($p=0.015$). In summary, our data show a novel leukemogenic mechanism through *SETBP1* overexpression; moreover, multivariate analysis confirms the negative prognostic impact of *SETBP1* overexpression in AML, especially in elderly patients, where it could be used as a predictive factor in any future clinical trials with PP2A activators.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal malignancy that predominantly affects middle-aged and elderly adults. The disease is characterized by a differentiation block in early progenitors, which leads to the accumulation of immature cells in bone marrow (BM) and peripheral blood. Over recent years, several genetic markers with prognostic impact in AML have been identified, leading to a better understanding of the biology of this disease and, in some cases, providing targets for molecular therapies.[1] Cytogenetic aberrations have been reported as the most important prognostic factors for survival and response to therapy in AML, allowing the identification of molecular markers that have greatly advanced our understanding of leukemogenesis;[2] nevertheless, the nature of alterations responsible for initiation or progression of the disease is mostly unknown. Recent attempts to identify initiating or progression mutations by extensively re-sequencing tyrosine kinase genes,[3,4] expression profiling studies,[5-7] array-based CGH and/or SNP,[8-11] and even by unbiased whole-genome sequencing,[12] confirm that AML results from multiple genetic alterations in hematopoietic stem cells, and suggest that we have not yet discovered most of the relevant aberrations that contribute to the pathogenesis of this disease.

The *ETV6* gene (12p13) encodes a transcription factor frequently rearranged in both myeloid and lymphoid leukemias. Translocation breakpoints are distributed throughout the gene, and *ETV6* contributes to the pathogenesis of leukemia by diverse molecular mechanisms that are only partially understood. In most cases, the translocations result in the generation of in-frame fusion genes between different domains of ETV6 and partner genes encoding either kinases or transcription factors (reviewed in Cools et al., 2002).[13] However, in some cases involving the 5' end of *ETV6*, functionally significant fusions could not be detected and a different leukemogenic mechanism has been described: the deregulation of the expression of oncogenes located close to the breakpoints.[13,14] This molecular mechanism, which has been described mainly in lymphoid leukemias and lymphomas, is an uncommon mechanism in myeloid leukemias, although some examples have been reported.[13,15]

Here, we describe a novel leukemogenic mechanism in a patient with AML and a t(12;18)(p13;q12) involving *ETV6*. The translocation resulted in overexpression of *SETBP1* (18q12), located close to the breakpoint. We show that SETBP1 overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein, and leading to the formation of a SETBP1-SET-PP2A complex that results in PP2A inhibition and, therefore, promotes the proliferation of leukemic cells. Moreover, we show that *SETBP1* overexpression is a recurrent molecular event associated with a significantly shorter overall survival in AML, especially in elderly patients.

Material and Methods

Case report

A 76-year-old caucasian man was diagnosed with myelodysplastic syndrome (MDS). Disease evaluation of the patient 3 years after the diagnosis showed anorexia, perspiration and loss of 7Kg. Laboratory findings at this moment were: Hb 10.9g/dL, white cell count (WCC) 38.2 $x10^9$/L with 55% blasts, and platelet count 251 $x10^9$/L; BM aspirate was hypercellular, showing 80% blasts. Flow cytometry showed positivity for CD34, CD13, CD33, CD11b, and HLA-DR. The patient was diagnosed as AML-M5. Karyotype showed two clones: 46,XY,t(12;18)(p13;q12)[77%]/47,idem,+19[23%]. The patient received standard induction chemotherapy for two months, and had partial remission at the next evaluation. Laboratory findings showed Hb 9.9 g/dL, WCC 25.5 $x10^9$/L with 32% blasts, and 260 $x10^9$/L platelets. Karyotype at this moment was: 46,XY,t(12;18)(p13;q12)[24%]/46,XY[76%]. The patient relapsed 2 months later and, eventually, died. Karyotype at relapse showed the two clones detected at diagnosis: 46,XY,t(12;18)(p13;q12)[32%]/47,idem,+19[68%].

Fluorescence in situ hybridization

BACs obtained from the Roswell Park Cancer Institute (Buffalo, NY) were used to map the breakpoints in the patient samples. The order of the probes is centromere-418C2-96B19-434C1-telomere (12p13), and centromere-840B16-937P23-252G8-941F5-telomere (18q12). Probes were labeled with SpectrumGreen-dUTP or SpectrumOrange-dUTP. Centromeric probes for chromosomes 12 and 18 were also used in FISH experiments. FISH was performed as previously described.[14]

Rapid amplification of cDNA ends

3´- and 5´-RACE-PCR were performed from total RNA using the GeneRacer kit (Invitrogen, Groningen, The Netherlands). 3´RACE-PCR (94ºC 30sec, 72ºC 2min30sec, 5 cycles; 94ºC 30sec, 70ºC 2min30sec, 5 cycles; 94ºC 30sec, 64ºC 30sec, 68ºC 2min30sec, 25 cycles) was performed using the primer ETV6-EX1F (exon 1) and a reverse primer from the kit. Nested-PCR was performed under the same conditions with ETV6-EX1Fn (exon 1). Products were cloned and sequenced. For 5´RACE the same PCR conditions were used, with primers ETV6-EX3R and ETV6-EX3Rn (exon 3) (Table S1).

Nucleic acid isolation and RT-PCR

Total DNA was isolated using the QIAmp DNA minikit, and total RNA using the RNeasy minikit (Qiagen, USA). cDNA was synthesized with SuperScriptIII Reverse Transcriptase (Invitrogen, The Netherlands). RT-PCR reactions were carried out with Ampli-Taq Gold DNA Polymerase (Applied Biosystems, USA) after optimizing cycling conditions for each primer pair. To confirm the presence of the fusion products, RT-PCR reactions were performed on patient RNA with

primers ETV6-EX1F2, and CR18-B and CR18-E (Table S1). Products were cloned and sequenced.

Real-time RT-PCR

Quantification of the expression of *SETBP1*, *SET* and *ETV6* was performed using TaqMan Gene Expression Assays (Applied Biosystems, USA) specific for each gene. *GAPDH* was used as internal control. A gene was considered overexpressed if its expression value was higher than the cut-off value established for each gene (mean+5SD), defined by the analysis of 10 normal BM samples.

Cell culture and transfection

HEL, K562, 32Dcl3, and HEK293 cell lines were grown at 37ºC in a 5% CO_2 atmosphere. HEK293 cells were maintained in Dulbecco´s minimum essential medium, HEL and K562 in RPMI-1640 (Gibco, Invitrogen Life Technologies, UK), and 32Dcl3 in RPMI-1640 + 10% conditioned medium of cell line WEHI-3B (DSM ACC 26). Media were supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (0.1 mg/ml). For transfection experiments, HEK293 cells were seeded into 10cm dishes and transfected with 60µL of Lipofectamine2000 (Invitrogen) and 12µg of the plasmids expressing SETBP1(SHR)-V5, SETBP1-GFP, SETBP1(SETBD)-GFP, SET-GFP or empty vector as control. HEL and 32Dcl3 cells were seeded in culture flasks and transfected using the Nucleofector System (solution V; protocol X-005 for HEL, and E-032 for 32Dcl3) (Amaxa), with 4 µg of plasmidic vectors or 75 nM *SET* siRNA pool designed and synthesized by Dharmacon.

Plasmids

Human *SET* cDNA was obtained by RT-PCR from K562 RNA using an upstream primer containing an EcoRI site followed by the first 19 nucleotides of *SET* cDNA, and a downstream primer containing the last 21 nucleotides of *SET* linked to a BamHI site. The EcoRI/BamHI digested PCR product was subcloned into the pEGFP-C2 vector leading to the pEGFPC2-SET construct. Human *SETBP1* cDNA was obtained by RT-PCR from peripheral blood, and subcloned into the vector pEGFP-C2 through XhoI/SalI sites, resulting in the pEGFPC2-SETBP1 construct. The region from aa 1,167 to the end of SETBP1 was obtained by digestion from the pEGFPC2-SETBP1 construct and subcloned into the vector pEGFP-C2 through HindIII/SacII sites, resulting in the pEGFPC2-SETBP1(SETBD) construct. The region from aa 449 to aa 857 of SETBP1 was obtained by PCR from the pEGFPC2-SETBP1 and subcloned into the pcDNA3.1V5/His vector, leading to the pcDNA3.1V5/His-SETBP1(SHR). All cloning procedures were verified by sequencing.

Immunoprecipitation and Western blotting

Cells were lysed in 100µL of Lysis buffer containing 1% Triton X-100 and protease inhibitors (Complete Mini, Roche). After incubation on ice (30min) protein extracts were clarified (12,000 xg, 15min, 4ºC), denatured and subjected to SDS-PAGE and Western-blot. Antibodies used were goat polyclonal anti-SET (Santa Cruz Biotechnology), mouse monoclonal anti-PP2A (clone 1D6, Upstate

Inc.), rabbit monoclonal anti-PP2A^{Y307} (Epitomics), rabbit polyclonal anti-GFP (Santa Cruz Biotechnology) and mouse monoclonal anti-βtubulin (Sigma). For IP, lysates were precleared (1h, 4ºC) on a rotating wheel and immunoprecipitated with antibody-bound Protein G-Sepharose (8h, 4ºC). After washings, IPs were subjected to SDS-PAGE and Western-blot. Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare).

Proliferation assay, viability and total cell counts

Cell proliferation was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Cell viability and total counts were measured in triplicate with the Nucleocounter (Biogen), and the results confirmed by Trypan Blue method.

PP2A assays

PP2A assays were performed with cell lysates (50µg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described,[16] except that we added the protease inhibitor cocktail complete (Roche) to the protein extraction mix.

Statistical analysis

Statistical analyses were performed using SPSS 15 for windows (SPSS Inc, Chicago Illinois). Overall survival (OS) was defined as the time from diagnosis to death due to any cause or end of follow-up. Disease-free survival (DFS) was defined as the time from complete remission until relapse or death. Event-free survival (EFS) was defined as the time from diagnosis until first event, in which failure to achieve complete remission, relapse, death or end of follow-up were considered events. OS, DFS, EFS were determined according to the Kaplan–Meier method and survival comparisons were done with the Log-rank (LR) test, if proportional hazard assumption was fulfilled, and Breslow (B) otherwise. The Cox proportional hazards model was used to assess patient's outcome for patient stratified groups of age. The hazard model was adjusted taking into consideration relevant parameters that included cytogenetic group, complete remission, and *SETBP1* overexpression. Good-and intermediate-risk were analyzed together because of the small number of patients with good-risk cytogenetics. $P < 0.05$ was considered statistically significant.

Bioinformatics analysis of the *SETBP1* proximal promoter

Analysis of the proximal promoter of *SETBP1* was performed with MotifScanner,[17-19] which scans DNA sequences with precompiled motif models. This algorithm assumes that motifs are hidden in a noisy background sequence represented by a higher-order Markov model. The motif models of transcriptional factors were obtained from the public version of Jaspar and Transfac databases. The proximal promoter of *SETBP1* was defined as 3,000bp upstream the

transcription start site (TSS), and was extracted from Ensembl database release 53.

Results

SETBP1 (18q21) is overexpressed in a patient with a translocation t(12;18) involving *ETV6*

Karyotype at diagnosis of a patient with AML-M5 secondary to MDS was 46,XY,t(12;18)(p13;q12)/47,idem,+19. FISH showed that the breakpoint on 12p13 was located between exons 2 and 3 of *ETV6* (Figure S1). To identify the fusion partner of *ETV6*, RACE-PCR experiments were performed on RNA from BM of the patient. 3'RACE-PCR identified two clones containing *ETV6* exon 2 followed by sequences that overlapped with the human cDNA clone BC051727 (Mammalian Gene Collection Program Team) located in chromosome 18 (Figure 1). Alignment of these sequences to the human genome did not reveal the presence of a known gene, but the novel sequences were split into segments that were flanked by consensus splice donor and acceptor sites. This indicated that these sequences could be part of a novel gene, whose exact identity and complete cDNA sequence was not further analyzed. Because the complete transcript is currently unknown, the different exons identified in this sequence were arbitrarily named as follows: Fusion transcript 1: *ETV6*-exon-1+*ETV6*-exon-2+exon-"a"+exon-"b"; Fusion transcript 2: *ETV6*-exon-1+*ETV6*-exon-2+exon-"c"+exon-"d"+exon-"e". Stop codons were found in the 3 different reading frames for exons "b" and "e", indicating that these exons are part of a noncoding transcript. The presence of these two fusions was confirmed by RT-PCR, cloned and sequenced (data not shown). Fusion transcripts predicted, in both cases, truncated proteins due to the presence of premature stop codons (Figure 1). Besides, the predicted new proteins showed no homology with any known proteins. In these novel sequences we found no microRNAs that could be deregulated as a consequence of the translocation. 5´RACE-PCR failed to find any reciprocal fusion transcript. Taken together, these data suggest that no functionally significant fusion transcripts were generated by the translocation.

To confirm the position of the breakpoint on chromosome 18, BACs located at 18q12 were used as probes in FISH experiments. Analysis on BM cells of the patient showed that one signal hybridized to the normal chromosome 18, and the other split and hybridized to both der(18) and der(12) (Figure 1). FISH showed that the breakpoint was located 5´ and close to the *SETBP1* gene. Activation of *SETBP1* expression by retroviral integration in hematopoietic progenitor cells has been reported to confer a growth advantage leading to clonal expansion.[20] Knowing that *SETBP1* was close to the breakpoint in the t(12;18)(p13;q12) and taking into account that ectopic expression of oncogenes is a mechanism involved in leukemia, we analyzed the expression of *SETBP1* by real-time PCR (QRT-PCR) in this patient, and we found that *SETBP1* was overexpressed at diagnosis and in the post-treatment samples (Figure 1).

Ectopic expression of SETBP1 leads to increased full-length SET protein levels

SETBP1 has been reported to specifically interact with the protein SET,[21] a potent inhibitor of protein phosphatase 2A (PP2A). To assess whether the ectopic expression of SETBP1 affects SET protein levels, HEK293 cells were transiently transfected with SETBP1-GFP. We found that levels of the 39KDa full length form of SET were strongly induced; moreover, an important decrease in the levels of low molecular weight forms of SET was observed. As controls, ectopic expression of the SETBP1-GFP protein was detected by anti-GFP (Figure 2A) and QRT-PCR (Figure S2A). Analysis by fluorescence microscopy of SETBP1-GFP-transfected HEK293 cells confirmed a predominantly nuclear location of SETBP1 (Figure S2B).

To assess the origin of the proteins of different sizes detected by anti-SET antibodies, HEK293 cells were transfected with SET-GFP and harvested at different times. Full-length SET-GFP protein of ~67KDa could be detected by western blot 9h after transfection, and two additional bands of lower molecular weight appeared gradually, together with the full-length form (Figure S2C). Taking into account the molecular weight of GFP, these bands would correspond to the endogenous short SET forms (Figure 2A). Because SET-GFP is expressed from a cDNA sequence, and alternative splicing cannot take place in the absence of intronic sequences, these results suggest that the endogenous SET protein of 39kDa is cleaved by proteases, resulting in truncated proteins with distinct sizes.

In order to analyze the region of SETBP1 that is critical to induce this effect, we transfected HEK293 cells with the vector pcDNA3.1-SETBP1(SHR)-V5, which expresses an internal region of SETBP1 that includes the SKI Homologous Region (SHR), and with the vector pEGFPC2-SETBP1(SETBD), expressing the carboxi-terminal region of SETBP1 that includes the SET binding domain (SETBD). We used HEK293 cells transfected with SETBP1-GFP as positive control, with the empty vector as negative control. We found similar results transfecting with SETBP1-GFP and with SETBD-GFP; however, no effects were observed with SETBP1(SHR)-V5, indicating that the region containing SETBD is critical (Figure 2B). Taken together, these results suggest that SETBP1 has a major effect on the 39kDa form of SET, probably increasing its levels by impairing protease activity on the full-length SET protein.

SETBP1 inhibits PP2A activity through tyrosine phosphorylation of its catalytic subunit

We next investigated the effect of SETBP1 overexpression on the PP2A protein. Expression levels of the catalytic subunit of PP2A (PP2Ac) were not affected in HEK293 cells transfected with SETBP1-GFP, with respect to non-transfected cells (Figure 2C). However, an increase in the phosphorylation of tyrosine-307 was observed (Figure 2D). Of note, phosphorylation of tyrosine-307 is responsible for more than 90% of the phosphatase activity of this protein; in fact, it has been shown that PP2Ac is inactive when tyrosine-307 is phosphorylated.[22] These results suggest that ectopic expression of SETBP1 leads to a reduced PP2A activity, which was confirmed by a PP2A phosphatase assay (Figure 2E).

We next assessed if the observations made in SETBP1-transfected cells could be confirmed in the patient samples. As a positive control for PP2A inactivation and SET overexpression, protein extracts from K562 cells were included.[23] The patient showed higher phosphorylation of tyrosine-307 of PP2Ac in both relapse and post-treatment samples, when compared with normal donors. We also observed increased SET protein levels, with good correlation between protein and mRNA (Figures 3 and S3).

SETBP1 induces proliferation in HEL and 32Dcl3

To investigate the effect of SETBP1 on cell growth, the HEL cell line (AML-M6) was chosen as a cellular model. An increased proliferation in cells transfected with SETBP1 compared with mock-transfected cells was observed using the MTS assay (Figure 4A); furthermore, total cell counts and cell viability were determined by Nucleo Counter and confirmed with the Trypan Blue method (data not shown). These results confirmed the higher cell counts and better viability of cells transfected with SETBP1 when compared to cells transfected with the empty vector. Similar results were obtained with the 32Dcl3 cell line (Figure 4A). In addition, we observed reduced PP2A activity in both HEL and 32Dcl3 cells transfected with SETBP1.

To confirm our hypothesis that SETBP1 inhibits PP2A through SET, SET was down-regulated by *SET*-specific siRNAs (Figure S4). We observed that the effects of SETBP1 on cell proliferation and PP2A activity were impaired in both HEL and 32Dcl3 cells after *SET* knockdown (Figure 4B).

SETBP1 forms a complex including SET and PP2A

It has been reported that SETBP1 interacts with SET and that the region of SET that binds to SETBP1 is different from the PP2A inhibitory region. Accordingly, it was postulated that SETBP1-SET heterodimers interact with PP2A.[21] However, no experimental evidence to confirm this hypothesis has been provided yet. To determine whether SET interacts simultaneously with SETBP1 and PP2A, coimmunoprecipitation assays were carried out in HEK293 cells, transfected or not with SETBP1-GFP. PP2A and SET coimmunoprecipitated with SETBP1, suggesting the existence of a SETBP1-SET-PP2A complex. Preclearing of SET using anti-SET antibodies bound to Sepharose-Protein G inhibited the coimmunoprecipitation of PP2A and SET with SETBP1, indicating that PP2A interacts with the SETBP1-SET heterodimer through SET, and that SETBP1 and PP2A do not directly interact with each other. Interestingly, we found that the coimmunoprecipitated PP2A was phosphorylated on its tyrosine-307 (Figure 5). Taken together, these results suggest that the SETBP1-SET heterodimer interacts with PP2A forming a SETBP1-SET-PP2A complex in which the presence of SET is critical.

Prevalence of *SETBP1* overexpression in AML

To study the prevalence of *SETBP1* overexpression and its prognostic value in AML, we quantified the expression of *SETBP1* in a series of 192 patients with

AML at diagnosis, correlated these values with FAB classification, and cytogenetic and molecular markers, and studied the prognostic relevance of this aberration. Patient characteristics are presented in Table 1, and box-plots showing the *SETBP1* expression levels in Figure S5. Although patients were treated with different schedules, all received regimes based on anthracycline and cytarabine as induction therapy. High dose cytarabine, and autologous or allogenic stem cell transplantation when possible, were used as consolidation therapy. All patients provided informed consent. *SETBP1* was overexpressed in 53 out of 192 patients (27.6%). The prevalence in cytogenetic prognostic groups was: good 24% (7/29), intermediate 18.5% (17/92), and poor 40.8% (29/71). We found genetic aberrations associated with *SETBP1* overexpression: monosomy 7 (p=0.007), chromosome 18 aberrations (p=0.031) and *EVI1* overexpression (p=0.001). An inverse correlation was observed between *SETBP1* overexpression and normal karyotype (p=0.010), and *NPM1* mutations in patients with wild type *FLT3* (p=0.045) (Table 2).

Prognostic impact of *SETBP1* overexpression in AML

Clinical follow-up data were available for 168 patients (Table S2), 91 men and 77 women, with a median age of 56 years. Median OS of the global cohort was 31.7 (95% CI 21.5-41.9 weeks) (Figure S5). As expected, significant differences in OS according to age, cytogenetic group, and complete remission rate (CR) were found in this series (p<0.01). In addition, we found significant differences in OS between patients with and without *SETBP1* overexpression (p<0.01) (Figure S6). Interestingly, the prognostic impact of *SETBP1* overexpression was particularly evident in patients older than 60 years (Figure 6A). Univariate analysis showed that only complete remission (p<0.01) and *SETBP1* overexpression (p=0.015), but not cytogenetic group (p=0.059), were significant in OS in the group of patients older than 60 years. Multivariate analysis demonstrated that *SETBP1* overexpression was an unfavorable independent factor associated with OS in elderly patients with AML (Table 3).

Only 121 of this series of 168 patients received induction therapy. We also found significant differences in age, cytogenetic group, and CR (data not shown) in this series. When we investigated the prognostic impact of *SETBP1* overexpression in this cohort, we found that patients with *SETBP1* overexpression had significantly worse OS (p=0.016) and EFS (p=0.015) (Figure 6B). We found no differences in DFS (p=0.366). Sixty six patients of this series were included in the intermediate cytogenetic risk group, and 47 had normal karyotype. Patients with *SETBP1* overexpression had a worse OS than those without (median: 26 vs. 45.9 weeks); however, differences were not statistically significant, probably due to the small number of *SETBP1* positive cases. A worse outcome in DFS (median: 20.9 vs. 61.4 weeks) and EFS (median: 23.3 vs. 40 weeks) was also observed.

In order to analyze the molecular events that could lead to *SETBP1* overexpression in the AML cases we performed a bioinformatic analysis of the proximal promoter of *SETBP1*. We identified several hypothetical binding-sites for transcription factors previously implicated in leukemia such as GATA1, GATA2, EVI1, HMG-1 or AP-1 (Table S3). Moreover, there is a CpG island within the putative proximal promoter of *SETBP1* that spans 1,812bp and could lead to the hypothetical epigenetic deregulation of *SETBP1* in cases with

overexpression of this gene. Interestingly, 6 out of the 7 retroviral insertion sites described by Ott et al. (2006)[20] are located in a region of 200pb (40513701-40513912), where this CpG island is located (40512982-40514793).

Discussion

We report here a novel mechanism of transformation in acute myeloid leukemia. We first investigated a t(12;18)(p13;q12) involving *ETV6* in a patient with AML. Functionally significant fusions could not be detected. Knowing that *SETBP1* (18q12) was close to the translocation breakpoint, and taking into account that ectopic expression of oncogenes is a mechanism involved in leukemia, we postulated that overexpression of *SETBP1* could be the major event in this case. In fact, *SETBP1* was overexpressed at diagnosis and in the post-treatment samples of the patient (Figure 1). As activation of *SETBP1* expression by retroviral integration in hematopoietic progenitor cells had been reported to confer a growth advantage leading to clonal expansion,[20] and we could establish that *SETBP1* overexpression was a recurrent molecular event with poor prognostic impact in AML, we decided to investigate the leukemogenic mechanism of overexpression of this gene. In this paper we show that SETBP1 overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein, and leading to the formation of a SETBP1-SET-PP2A complex that results in PP2A inhibition, and therefore promotes the proliferation and expansion of leukemic cells.

SETBP1 encodes a protein of 1,542 aa and 170KDa, localized predominantly in the nucleus, as we have confirmed in this study (Figure S2B). The physiological function as well as the molecular mechanism by which SETBP1 acts remains unknown. The protein contains a region homologous to the dimerization domain of SKI, and a SET binding region, although the functional significance of these interactions has not been determined.[21] The protein SET (I2PP2A/TAF-Iβ) inhibits PP2A through the phosphorylation of the PP2Ac tyrosine 307.[24] In fact, SET is overexpressed in multiple solid tumors[25] and in CML, where its correlation has been demonstrated with the expression and oncogenic activity of BCR-ABL, leading to PP2A inhibition.[23]

Here, we demonstrate that SETBP1 overexpression increased the 39KDa full-length SET, probably because the SETBP1-SET interaction protects SET from protease-mediated cleavage. As a consequence, we detected a decrease in the 27KDa, 24KDa and 20KDa SET forms (Figure 2A). It has been reported that SET inhibits the DNase activity of the tumor suppressor NM23-H1, and that the cleavage of SET by Granzyme A during the cytotoxic T lymphocyte-induced apoptosis releases NM23-H1 from inhibition, and triggers NM23-H1 to translocate into the nucleus, where it nicks the DNA.[26,27] Interestingly, the cleavage of SET by Granzyme A generates 3 polypeptides with similar molecular weight to those we detected. Although more studies are needed to clarify this point, our results suggest a possible role for SETBP1 impairing the cleavage of SET by Granzyme A in the apoptosis caspase-independent pathway induced by cytotoxic T lymphocytes. This could point to a novel defense mechanism in leukemic cells.

PP2A is a major protein phosphatase implicated in many cell processes,[28-32] and its loss of function has been associated with cell transformation.[33,34] Because SET is a potent inhibitor of PP2A, and SETBP1 overexpression alters the level of SET, we analyzed the effect of SETBP1 overexpression on PP2A. In addition to the previously reported SETBP1-SET and SET-PP2A complexes, we demonstrated

the formation of a SETBP1-SET-PP2A heterotrimeric complex, in which PP2A is phosphorylated and, therefore, inhibited (Figure 5). Our results suggest that this is the molecular mechanism by which PP2A is inhibited in patients with *SETBP1* overexpression and AML, since PP2A and SETBP1 did not interact in the absence of SET (Figure 5). Interestingly, we only detected the full-length SET form in the immunoprecipitate, indicating that the shorter processed forms are not present in the SETBP1-SET-PP2A complex. It has been reported that these forms conserve their inhibitory activity of PP2A, but it remains unclear whether this activity is similar to that of full-length SET;[35] our results suggest that the processed forms may have lower PP2A inhibitory activity, and that SETBP1 may increase the inhibition of PP2A by protecting the full-length SET protein, and permitting its interaction with PP2A.

Moreover, SET is a key modulator of DNA replication, chromatin remodeling and gene transcription,[36,37] differentiation,[38] and cell-cycle regulation.[39] We postulate that the protecting role that SETBP1 overexpression plays on SET might reflect changes in the expression patterns of genes whose acetylation depends on the presence of the full-length SET protein, since 39KDa SET, but not the processed forms, has been reported to inhibit histone acetylation and to bind to HuR, which stabilizes immediate early gene mRNAs.[25,36,40] Our results showed that the SETBD region is critical for this function (Figure 2B); therefore, the interaction of SETBP1 and SET could have other effects apart from the inhibition of PP2A.

To assess the prevalence and the prognostic significance of *SETBP1* overexpression we analyzed the expression of this gene in 192 patients with AML at diagnosis, and we found that *SETBP1* overexpression was a recurrent event in AML, accounting for 27.6% of all cases. Our results show that *SETBP1* overexpression predicts shorter OS, and that the impact on prognosis is especially significant in patients older than 60 years (Figure 6A). AML is a disease of the elderly, with a median age at diagnosis over 60 years. However, the gradual improvements achieved in the last two decades have been mainly focused on the group of patients <60, whereas there has been no change in the OS of patients >60, probably due to both patient and disease specific factors, although this subgroup represents two thirds of the total number of cases.[41] It is therefore important to identify genetic markers that could predict prognosis in this subgroup of patients, as well as to advance our knowledge of disease biology to develop novel targeted therapies. Our data suggest that *SETBP1* overexpression could distinguish two subgroups of AML elderly patients; furthermore, it could be a predictive factor of response to PP2A activators such as FTY720, which has been proposed as a future alternative for treating blast crisis CML and Philadelphia-positive ALL.[42]

Although multivariate analysis confirmed the negative prognostic impact of *SETBP1* overexpression in our series, it was associated with other poor prognostic markers such as monosomy 7 and *EVI1* overexpression. AML is a multistep genetic disease, and the activation of *SETBP1* may cooperate with additional mutations to induce leukemia.[20] In the patient with t(12;18) we found no *FLT3*, *NPM1* or *JAK2* mutations, although trisomy 19 was observed in the blast cells of the patient as a secondary event that would cooperate in the progression of the disease. Moreover, post-treatment samples showed overexpression of *SET* and

ETV6, which could act synergistically with *SETBP1* overexpression (Figure S3). The mechanisms by which *SETBP1* is overexpressed in the series analyzed remain unknown. However, bioinformatic analysis of the putative promoter region of *SETBP1* allowed us to identify several transcription factors previously implicated in leukemia (Table S3); so, we postulate that their deregulation could promote *SETBP1* overexpression. FISH analysis suggests that the breakpoint in the patient with the t(12;18) is located upstream to the transcription start site of *SETBP1*, close to the retroviral insertion sites described by Ott et al. (2006)[20]. Moreover, 6 out of the 7 retroviral insertion sites described in that paper are located in a region of 200bp (40513701-40513912), where we have found a CpG island (40512982-40514793). Taken together, this suggests that this is an important region for the transcriptional regulation of the *SETBP1* gene, and that epigenetic aberrations could be another mechanism of *SETBP1* overexpression in patients with AML without 18q aberrations. Further studies to confirm this and to analyze the promoter region of *SETBP1* are in progress.

In summary, we report a novel mechanism of leukemic transformation. Overexpression of SETBP1 promotes an increase in full-length SET levels, impairing the phosphatase activity of the tumor suppressor PP2A through the formation of a SETBP1-SET-PP2A complex, and promoting the proliferation of cells (Figure 7). Besides, we demonstrate that SETBP1 overexpression protects SET from protease cleavage, which could have important effects on both the histone acetylation inhibitory activity of SET and Granzyme A-mediated caspase-independent apoptosis induced by cytotoxic T lymphocytes. Furthermore, deregulation of *SETBP1* by translocations or other unknown mechanisms seems to play a crucial role in the leukemic transformation of AML. We have shown that *SETBP1* overexpression is a recurrent molecular event with independent prognostic value in AML, especially in the subgroup of elderly patients. Further research into the physiological function of this gene will contribute to a better understanding of the multiple steps that give rise to AML.

Acknowledgments: This work was supported by the Ministerio de Educación y Ciencia (AP2006-03038), Ministerio de Ciencia e Innovación (PI081687 to MDO and SAF2007-61827 to CB), Departamento de Salud del Gobierno de Navarra (14/2008), ISCIII-RETIC (RD06/0020/0078), and Fundación para la Investigación Médica Aplicada y UTE (Spain). We thank Elizabeth Guruceaga, of the Unit of Proteomics, Genomics and Bioinformatics of CIMA, for the bioinformatics analysis, and Enrique Andreu for useful discussion.

Authorship Contributions: IC, FJB, LGO and NM performed research; CV performed FISH analyses; FJN and EB contributed to bioinformatics and statistical analyses; JR and MJC made available clinical histories and patient samples; IC, CB and MDO designed research and wrote the paper.

Conflict of interest disclosure: No potential conflicts of interest were disclosed.

Tables

Table 1. Clinical and molecular characteristics of a series of 192 patients with AML.

		No. (%)	
Sex			
	Male	99	(53)
	Female	88	(47)
	No data	5	
Age			
	<60 years	92	(50.3)
	>60 years	91	(49.7)
	No data	9	
Complete Remission			
	No	47	(36.7)
	Yes	81	(63.3)
	No data	64	
Diagnosis			
	AML-M0	21	(10.9)
	AML-M1	35	(18.2)
	AML-M2	38	(19.8)
	AML-M3	7	(3.6)
	AML-M4	33	(17.2)
	AML-M5	36	(18.8)
	AML-M6	12	(6.3)
	AML-NOS	10	(5.2)
Secondary AML (sAML)			
	No	130	(83.3)
	Yes	26	(16.7)
	No data	36	
Cytogenetic group			
	good	29	(15)
	intermediate	92	(48)
	poor	71	(37)
SETBP1 overexpression			
	No	139	(72.4)
	Yes	53	(27.6)
WT1 overexpression			
	No	22	(16)
	Yes	115	(84)
	No data	55	
EVI1 overexpression			
	No	132	(76.7)
	Yes	40	(23.3)
	No data	20	
NPM1 mutated			
	No	18	(37.5)
	Yes	30	(62.5)
	No data	144	
FLT3-ITD			
	No	99	(79.2)
	Yes	26	(20.8)
	No data	67	

Table 2. Association between *SETBP1* overexpression and clinical and genetic parameters in 192 patients with AML at diagnosis.

		No. Cases	**No. SETBP1- (%)**		**No. SETBP1+ (%)**		**P**
SETBP1		192	139 (72.4)		53 (27.6)		
Sex		187	136		51		0.511
Male		99	70	(70.7)	29	(29.3)	
Female		88	66	(75)	22	(25)	
Age		183	136		47		0.219
<60 years		92	72	(78.3)	20	(21.7)	
≥ 60 years		91	64	(70.3)	27	(29.7)	
CR		128	180		50		0.196
No		47	33	(70.2)	14	(29.8)	
Yes		81	65	(80.2)	16	(19.8)	
sAML		156	247		56		0.354
No		130	101	(77.7)	29	(22.3)	
Yes		26	18	(69.2)	8	(30.8)	
Prognostic group		192	139		53		**0.006**
good		29	22	(76)	7	(24)	
intermediate		92	75	(82.5)	17	(18.5)	
poor		71	42	(59.2)	29	(40.8)	
Cytogenetic group		192	139		53		
normal karyotype	yes	63	53	(84)	10	(16)	**0.010**
	no	128	85	(66.4)	43	(33.6)	
trisomy 8	yes	18	13	(72.2)	5	(27.8)	0.943
	no	163	119	(73)	44	(27)	
chromosome 18 aberrations	yes	11	5	(45.4)	6	(54.6)	**0.031**
	no	169	127	(75)	42	(25)	
monosomy 7	yes	27	14	(51.9)	13	(48.1)	**0.007**
	no	155	119	(76.8)	36	(23.2)	
der(7q)	yes	16	10	(62.5)	6	(37.5)	0.318
	no	166	123	(74.1)	43	(25.9)	
WT1 overexpression		137	108		29		0.345
No		22	19	(86.7)	3	(13.6)	
Yes		115	89	(77.4)	26	(22.6)	
EVI1 overexpression		172	126		46		**0.001**
No		132	108	(81.8)	24	(18.2)	
Yes		40	18	(45)	22	(55)	
MDS1EVI1 overexpression		152	109		43		0.012
No		138	103	(74.6)	35	(25.4)	
Yes		14	6	(42.9)	8	(57.1)	
NPM1 mutated and FLT3 wt		32	28		4		**0.014**
No		12	8	(66.7)	4	(33.3)	
Yes		20	20	(100)	0	(0.0)	
FLT3-ITD		125	100		25		0.760
No		99	78	(78.8)	21	(21.2)	
Yes		26	22	(84.6)	4	(15.4)	

Table 3. Multivariate analysis of clinical and biological variables in the group of patients with AML older than 60 years.

Variable	Subset	Hazard ratio (95% confidence interval)	p
Complete Remission	No/Yes	1 / 0.218 (0.110 - 0.430)	0.001
SETBP1 overexpression	No/Yes	1 / 2.311 (1.173 - 4.553)	0.015

Figures

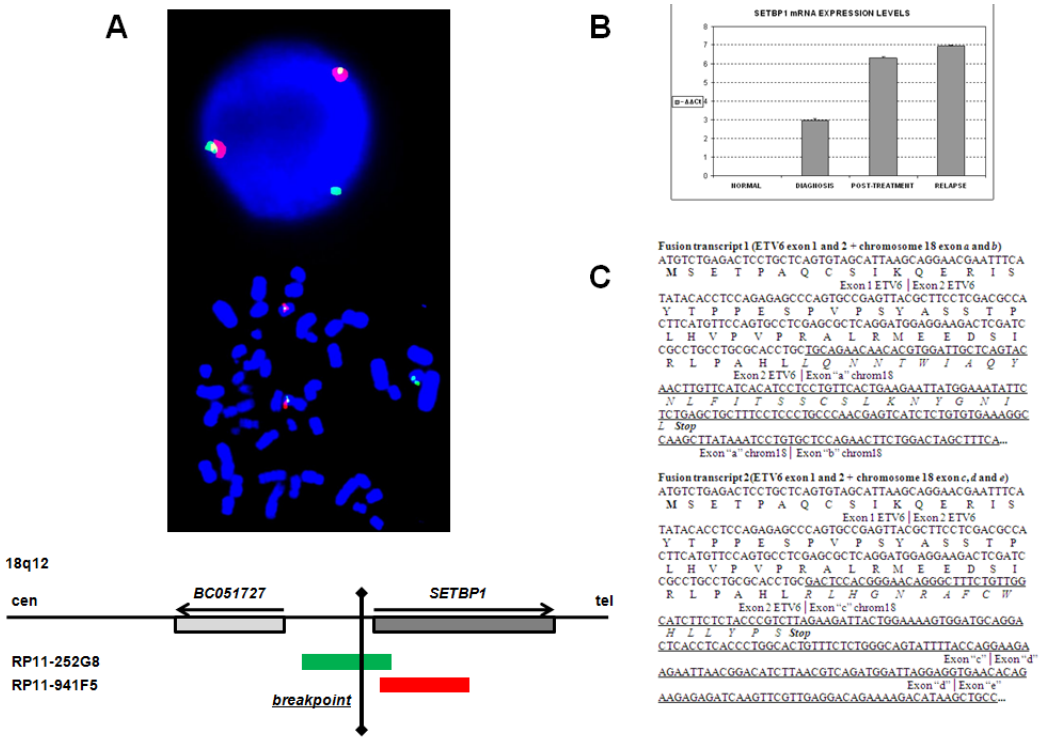


Figure 1. Genetic characterization of a patient with AML-M5 and a t(12;18)(p13;q12) involving *ETV6*. A) FISH analysis indicating the breakpoint on 18q12: probe RP11-252G8 (green) splits and hybridizes in both der(18) and der(12); B) Analysis by QRT-PCR of the *SETBP1* expression in samples of the patient at diagnosis, post-treatment control, and relapse; C) RACE results showing the two different fusion transcripts detected. Sequences from *ETV6* are in italics; the putative exons from chromosome 18 are underlined.

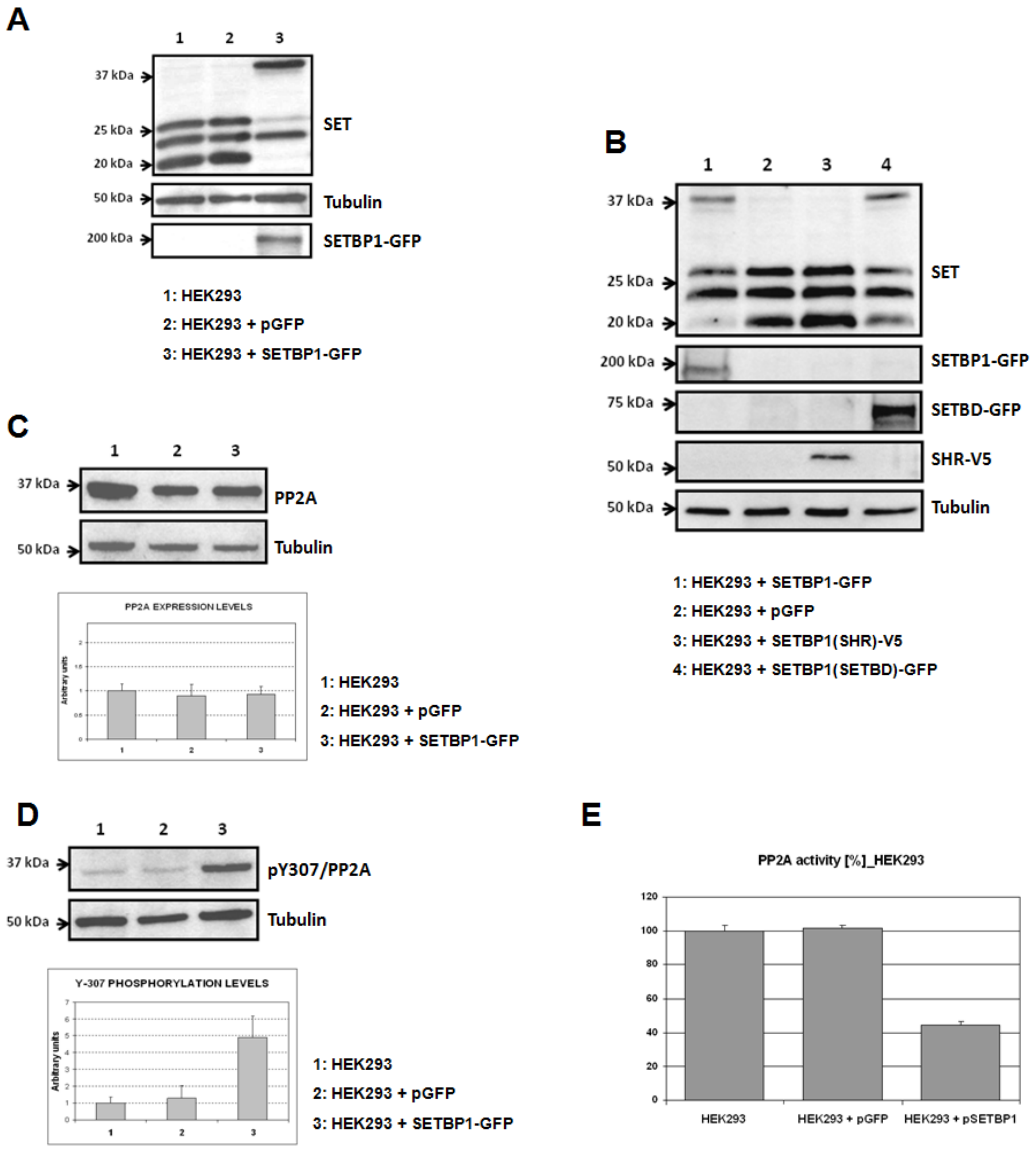


Figure 2. Ectopic expression of SETBP1 induces increased full-length SET levels and inhibits PP2Ac. A) Western-blot showing endogenous SET expression in HEK293 cells transfected with SETBP1-GFP, an empty vector as a control, or non-transfected cells; B) Analysis by western-blot showing SET in HEK293 cells transfected with different regions of SETBP1; SHR: SKI homologous region; SETBD: SET binding domain; C) Western-blot and densitometry analysis showing the effect of ectopic SETBP1 over PP2A expression in HEK293 cells; D) Western-blot and densitometry analysis showing the effect of ectopic SETBP1 over PP2A phosphorylation levels of the tyrosine-307; E) PP2A phosphatase assay in HEK293 cells transfected or not with SETBP1.

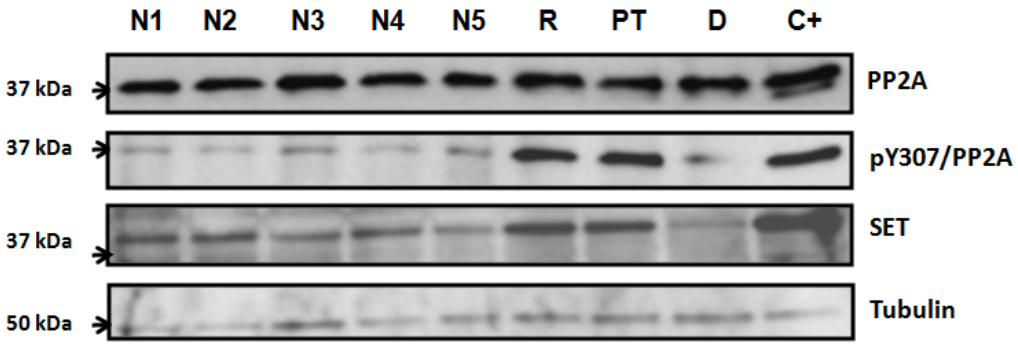


Figure 3. Comparison of the PP2A and SET protein levels by western-blot between patient samples and normal donors. Phosphorylation on tyrosine-307 of PP2A is also assessed. The K562 cell line was used as a positive control for SET overexpression and hyperphosphorylation of Y-307 of PP2A. N1-N5: normal controls; R: patient sample at relapse; PT: patient sample after-treatment; D: patient sample at diagnosis; C+: K562 cells as positive control.

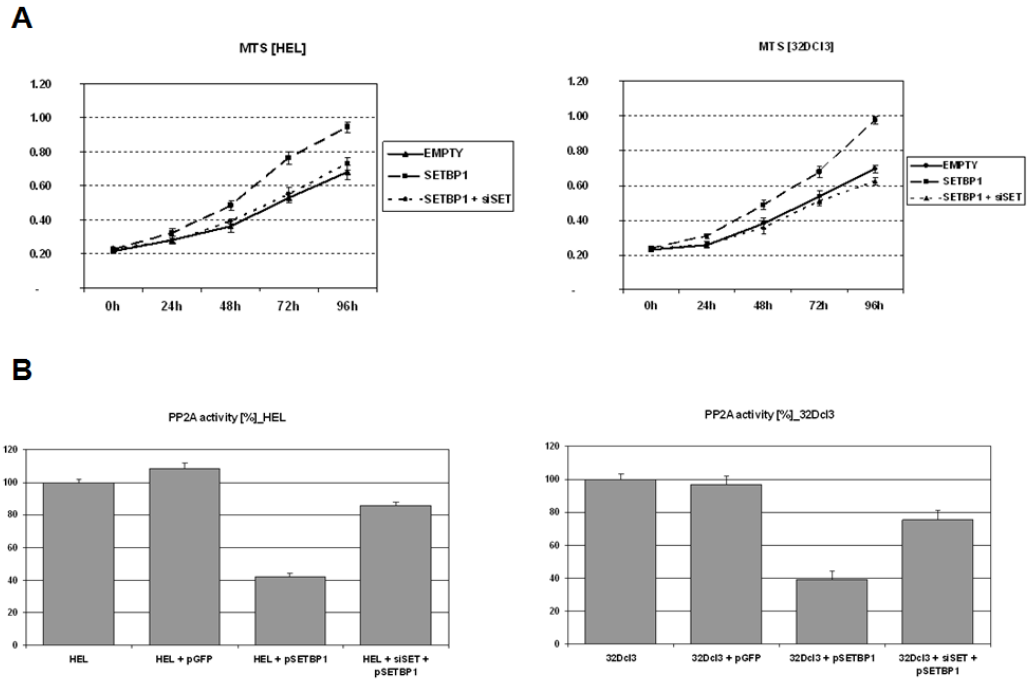


Figure 4. Effect of SETBP1 on cell proliferation and PP2A activity. A) MTS assays showing the effect of ectopic SETBP1 expression alone or with SET-specific siRNAs on the growth of HEL and 32Dcl3; B) PP2A phosphatase assay in HEL and 32Dcl3 cells either not transfected or expressing the empty vector as a control, SETBP1 alone or SETBP1 with SET downregulated by *SET*-specific siRNAs.

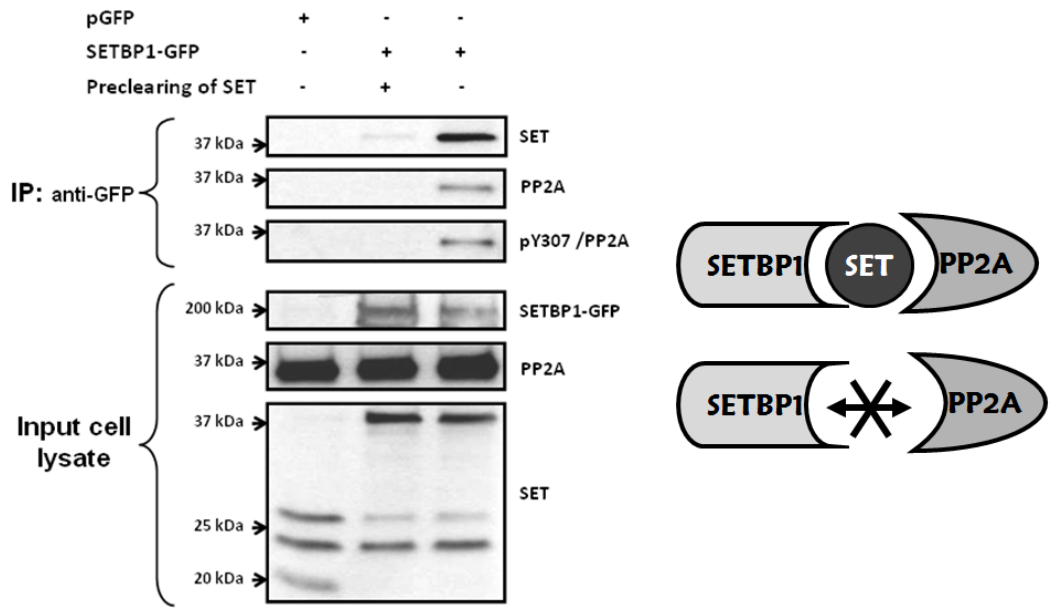


Figure 5. PP2A interacts with SET-SETBP1 heterodimer but not directly with SETBP1. Cell extracts expressing SETBP1-GFP (lanes 2 and 3) were immunoprecipitated with anti-GFP followed by western-blotting with anti-SET and anti-PP2A. Cell extract without SETBP1-GFP expression was used as negative control (lane 1). One of the cell extracts expressing SETBP1-GFP was pre-cleared of SET using anti-SET antibodies bound to Sepharose-Protein G before the immunoprecipitation (lane 2).

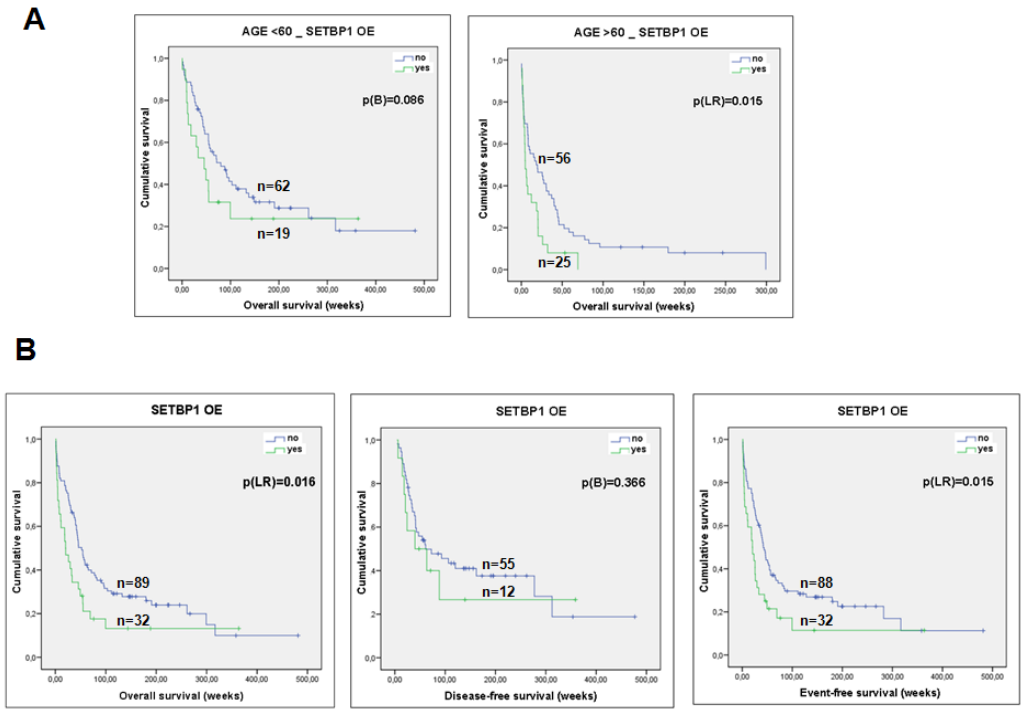


Figure 6. A) Kaplan-Meier analyses of overall survival for *SETBP1* in the series of age-stratified 168 patients with AML, and clinical follow-up data available. Patients older than 60 years with *SETBP1* overexpression showed a poorer outcome in comparison with patients with no *SETBP1* overexpression; B) Kaplan-Meier analyses of overall survival, disease-free survival and event-free survival for *SETBP1* overexpression in a series of 121 patients with AML and clinical follow-up data available who received induction therapy (note that this group of 121 patients is included in the previous series of 168 patients mentioned above).

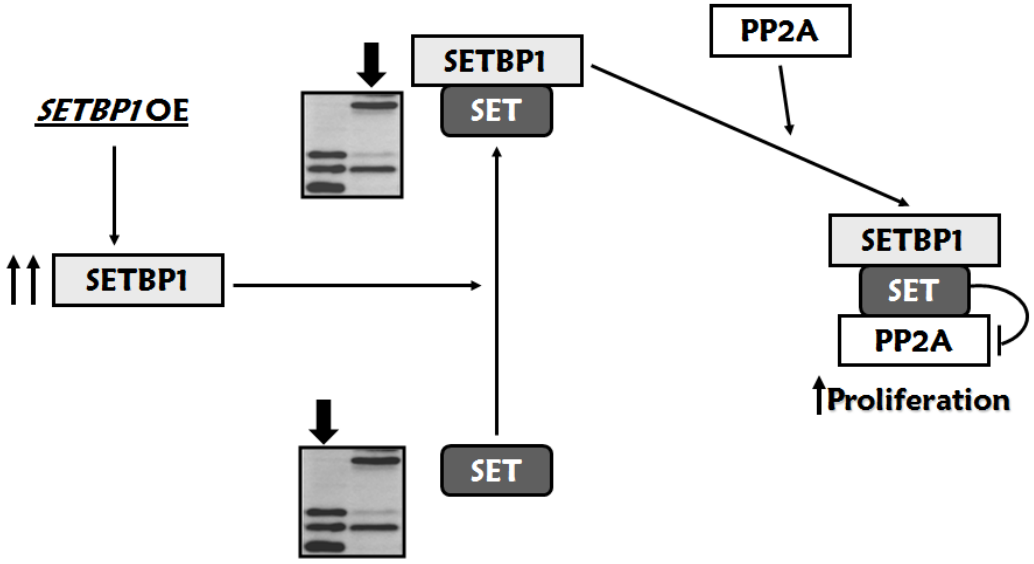


Figure 7. Proposed molecular model for SETBP1 signaling pathway: SETBP1 overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein, and leading to the formation of a SETBP1-SET-PP2A complex that results in PP2A inhibition and, therefore, promotes the proliferation of leukemic cells.

References

1. Dohner H. Implication of the molecular characterization of acute myeloid leukemia. Hematology Am Soc Hematol Educ Program. 2007:412-419.

2. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev. 2004;18:115-136.

3. Loriaux MM, Levine RL, Tyner JW, et al. High-throughput sequence analysis of the tyrosine kinome in acute myeloid leukemia. Blood. 2008;111:4788-4796.

4. Tomasson MH, Xiang Z, Walgren R, et al. Somatic mutations and germline sequence variants in the expressed tyrosine kinase genes of patients with de novo acute myeloid leukemia. Blood. 2008;111:4797-4808.

5. Schoch C, Kohlmann A, Schnittger S, et al. Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. Proc Natl Acad Sci U S A. 2002;99:10008-10013.

6. Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. N Engl J Med. 2004;350:1605-1616.

7. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004;350:1617-1628.

8. Raghavan M, Lillington DM, Skoulakis S, et al. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. Cancer Res. 2005;65:375-378.

9. Paulsson K, Heidenblad M, Strombeck B, et al. High-resolution genome-wide array-based comparative genome hybridization reveals cryptic chromosome changes in AML and MDS cases with trisomy 8 as the sole cytogenetic aberration. Leukemia. 2006;20:840-846.

10. Rucker FG, Bullinger L, Schwaenen C, et al. Disclosure of candidate genes in acute myeloid leukemia with complex karyotypes using microarray-based molecular characterization. J Clin Oncol. 2006;24:3887-3894.

11. Suela J, Alvarez S, Cifuentes F, et al. DNA profiling analysis of 100 consecutive de novo acute myeloid leukemia cases reveals patterns of genomic instability that affect all cytogenetic risk groups. Leukemia. 2007;21:1224-1231.

12. Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008;456:66-72.

13. Cools J, Mentens N, Odero MD, et al. Evidence for position effects as a variant ETV6-mediated leukemogenic mechanism in myeloid leukemias with a t(4;12)(q11-q12;p13) or t(5;12)(q31;p13). Blood. 2002;99:1776-1784.

14. Odero MD, Vizmanos JL, Roman JP, et al. A novel gene, MDS2, is fused to ETV6/TEL in a t(1;12)(p36.1;p13) in a patient with myelodysplastic syndrome. Genes Chromosomes Cancer. 2002;35:11-19.

15. Nucifora G, Laricchia-Robbio L, Senyuk V. EVI1 and hematopoietic disorders: history and perspectives. Gene. 2006;368:1-11.

16. Gallay N, Dos Santos C, Cuzin L, et al. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. Leukemia. 2009;23:1029-1038.

17. Coessens B, Thijs G, Aerts S, et al. INCLUSive: A web portal and service registry for microarray and regulatory sequence analysis. Nucleic Acids Res. 2003;31:3468-3470.

18. Bryne JC, Valen E, Tang MH, et al. JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. Nucleic Acids Res. 2008;36:D102-106.

19. Matys V, Kel-Margoulis OV, Fricke E, et al. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res. 2006;34:D108-110.

20. Ott MG, Schmidt M, Schwarzwaelder K, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. Nat Med. 2006;12:401-409.

21. Minakuchi M, Kakazu N, Gorrin-Rivas MJ, et al. Identification and characterization of SEB, a novel protein that binds to the acute undifferentiated leukemia-associated protein SET. Eur J Biochem. 2001;268:1340-1351.

22. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. Science. 1992;257:1261-1264.

23. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR-ABL-regulated SET protein. Cancer Cell. 2005;8:355-368.

24. Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. J Biol Chem. 1996;271:11059-11062.

25. Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. J Biol Chem. 2002;277:25026-25031.

26. Beresford PJ, Zhang D, Oh DY, et al. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. J Biol Chem. 2001;276:43285-43293.

27. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell. 2003;112:659-672.

28. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J. 2001;353:417-439.

29. Wang GL, Iakova P, Wilde M, Awad S, Timchenko NA. Liver tumors escape negative control of proliferation via PI3K/Akt-mediated block of C/EBP alpha growth inhibitory activity. Genes Dev. 2004;18:912-925.

30. Kawabe T, Muslin AJ, Korsmeyer SJ. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. Nature. 1997;385:454-458.

31. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell. 2007;130:21-24.

32. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med. 2008;14:152-160.

33. Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. Curr Opin Genet Dev. 2005;15:34-41.

34. Schonthal AH. Role of serine/threonine protein phosphatase 2A in cancer. Cancer Lett. 2001;170:1-13.

35. Beresford PJ, Xia Z, Greenberg AH, Lieberman J. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. Immunity. 1999;10:585-594.

36. Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. Cell. 2001;104:119-130.

37. Kutney SN, Hong R, Macfarlan T, Chakravarti D. A signaling role of histone-binding proteins and INHAT subunits pp32 and Set/TAF-Ibeta in integrating chromatin hypoacetylation and transcriptional repression. J Biol Chem. 2004;279:30850-30855.

38. Kandilci A, Mientjes E, Grosveld G. Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937. Leukemia. 2004;18:337-340.

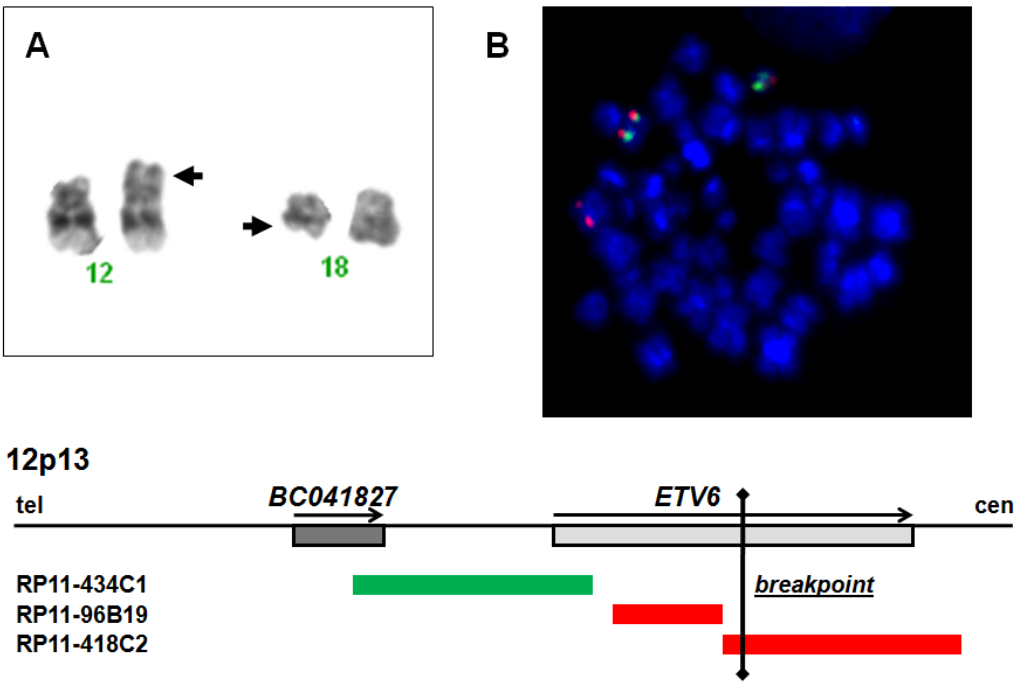
39. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, et al. The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. J Biol Chem. 2003;278:1158-1164.

40. Brennan CM, Gallouzi IE, Steitz JA. Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J Cell Biol. 2000;151:1-14.

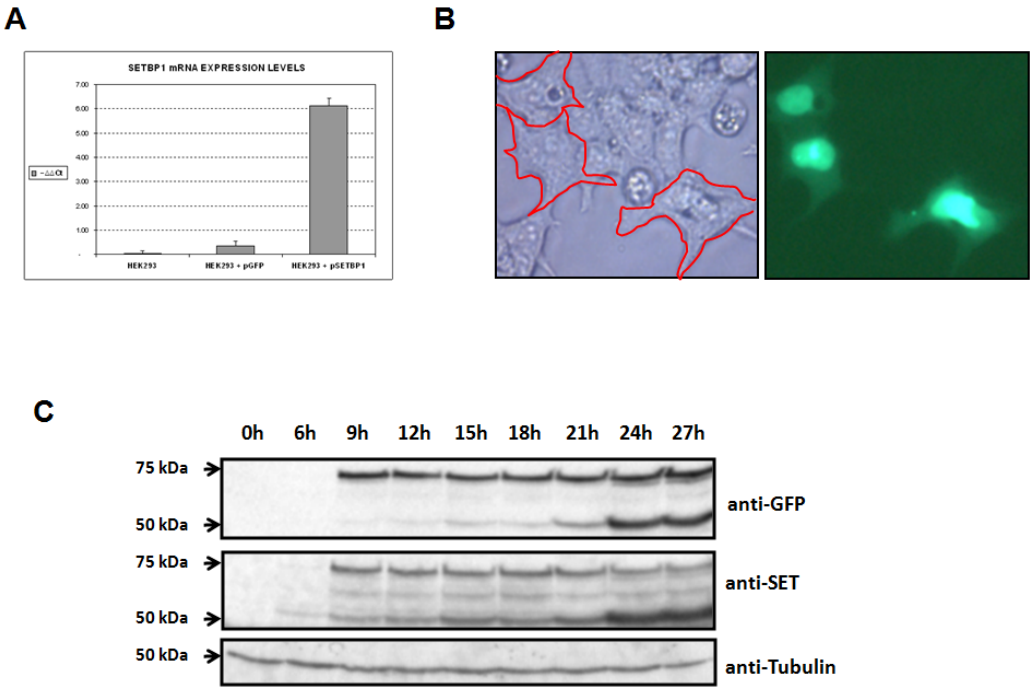
41. Buchner T, Berdel WE, Haferlach C, et al. Age-related risk profile and chemotherapy dose response in acute myeloid leukemia: a study by the German Acute Myeloid Leukemia Cooperative Group. J Clin Oncol. 2009;27:61-69.

42. Neviani P, Santhanam R, Oaks JJ, et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. J Clin Invest. 2007;117:2408-2421.

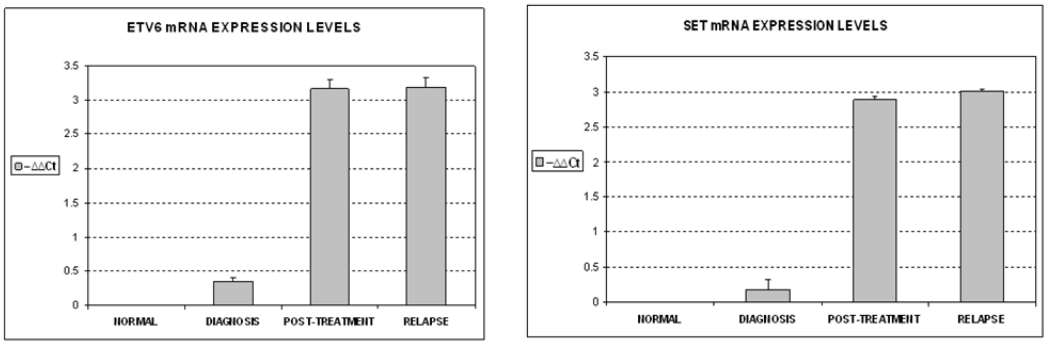
Supplementary Information



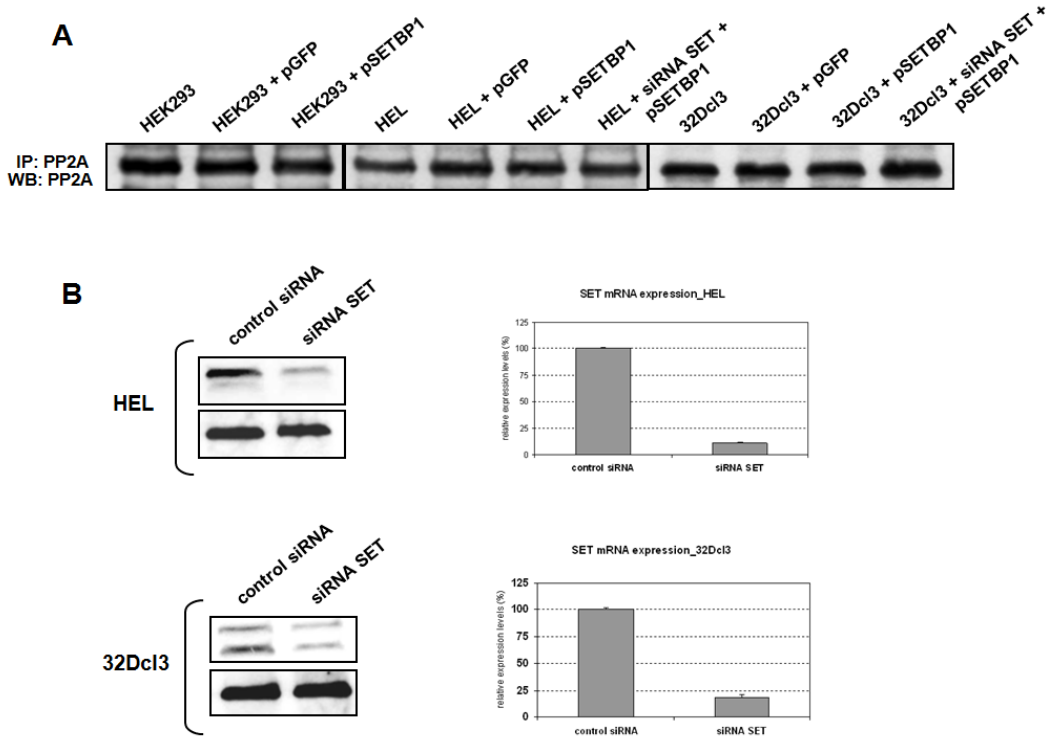
Supplementary Figure 1. Genetic characterization of a patient with AML-M5 and a t(12;18)(p13;q12). A) Partial karyotype of the patient. Derivative chromosomes are indicated by arrows; B) FISH analysis of the patient showing that *ETV6* was rearranged.



Supplementary Figure 2. A) Quantification of *SETBP1* expression levels by QRT-PCR (ΔΔCt method) in parental HEK293 cells and HEK293 cells expressing *SETBP1* or the empty vector. B) SETBP1 shows mainly nuclear intracellular location. Results were obtained using a Leica DMIRB fluorescence microscope at 100X magnification and a DC480 camera (Leica) and analyzed by the use of the TWAIN Module software (Leica). C) HEK293 cells were transiently transfected with SET-GFP and harvested at different times. SET-GFP expression began 9 hours after transfection, with two shorter forms were appearing progressively.

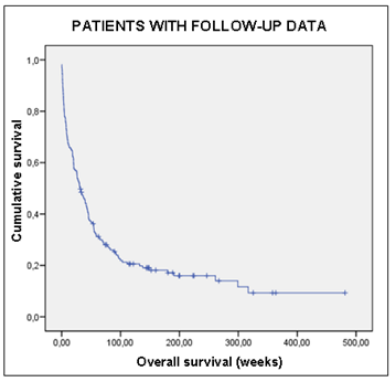


Supplementary Figure 3. Quantification of *SET* and *ETV6* expression levels by QRT-PCR (ΔΔCt method) in the patient samples at diagnosis, post-treatment control and relapse.



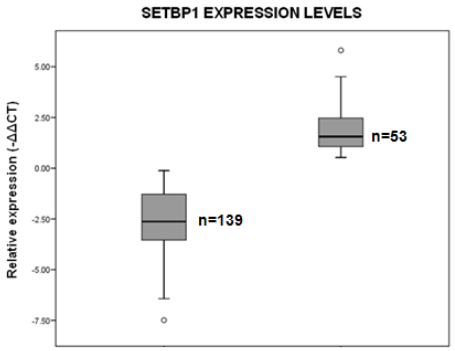
Supplementary Figure 4. A) Western blot analysis showing the levels of immunoprecipitated PP2A from the cell lines used in the phosphatase assays showed in fig. 2E and 4B; B) Western blot analysis and relative *SET* expression by QRT-PCR of HEL and 32Dcl3 cells transfected with a control siRNA or si*SET*.

A

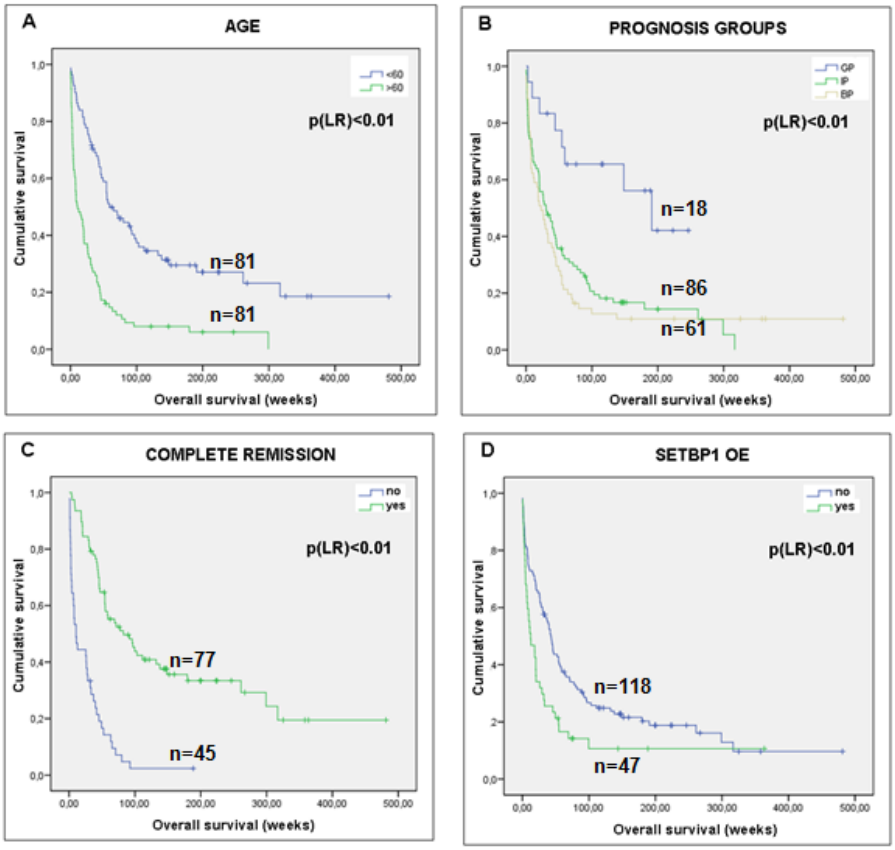


Time (weeks)	Cumulative Proportion Surviving at the time		Nº of Cumulative Events	Cases at risk
	Estimate	Std. Error		
26	55.1%	3.8%	76	92
52	36.8%	3.8%	108	60
104	21.2%	3.3%	137	31
156	11.4%	3.1%	149	19
206	6.6%	3.2%	157	11
260	4.8%	3.2%	160	8

B



Supplementary Figure 5. A) Survival graph of the 168 AML patients with follow-up data available; B) Box-plots showing *SETBP1* expression levels in the groups of AML patients without (n=139), and with *SETBP1* overexpression (n=53).



Supplementary Figure 6. Kaplan-Meier analyses of overall survival in the cohort of 168 patients with clinical follow-up data available show an inferior outcome in: A) patients older than 60 years; B) patients included in the intermediate and poor prognosis groups; C) patients who did not achieve CR; D) and patients with *SETBP1* overexpression.

Capítulo 4. Deregulation of *SET* is a recurrent event that contributes to PP2A inhibition and is associated with poor outcome in acute myeloid leukemia

(artículo científico enviado para su publicación a la revista *Blood*)

Deregulation of *SET* is a recurrent event that contributes to PP2A inhibition and is associated with poor outcome in acute myeloid leukemia

Ion Cristóbal,[1,2] Laura Garcia-Orti,[1] Cristina Cirauqui,[1] María A. García-Sánchez,[1] María J. Calasanz,[2] María D. Odero[1,2]

[1]Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain

[2]Department of Genetics, University of Navarra, Pamplona, Spain

Keywords: SET, PP2A, AML, prognosis

Running Title: SET deregulation in AML

Scientific category: Myeloid Neoplasia

Corresponding author: María D. Odero, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pio XII-55 31008-Pamplona, Spain. E-mail: modero@unav.es. Phone: +34-948-194700. Fax: +34-948-194714.

Abstract

PP2A is a tumor suppressor reported as a potential therapeutic target in chronic and acute leukemias. Here we show that SET, a potent PP2A inhibitor implicated in numerous cell processes, is upregulated in acute myeloid leukemia (AML) at both mRNA and protein levels. Prevalence of *SET* overexpression in AML patients at diagnosis was 28% (60/214), and was associated with *SETBP1* ($p<0.01$) and *EVI1* overexpression ($p=0.02$). Patients with *SET* overexpression had worse OS ($p<0.01$) and EFS ($p<0.01$). SET induces proliferation of AML cells, restoring the reduced cell viability caused by PP2A expression. Moreover, activation of PP2A by forskolin decreases SET, indicating that PP2A could regulate SET levels. Our results suggest that *SET* overexpression is a recurrent molecular event that predicts poor outcome, and represents a key mechanism to inhibit PP2A in AML. Importantly, this alteration discriminates a subgroup of AML patients that could benefit from future treatments with PP2A activators.

Introduction

Acute myeloid leukemia (AML) is a clonal disorder with great heterogeneity at clinical and molecular levels. Identification of recurrent genetic alterations has provided novel targets to improve prognosis and develop more effective therapies in AML; however, advances in our understanding of the pathophysiology of AML have not yet lead to major improvements in patient outcomes.[1-2] The protein SET (I2PP2A/TAF-Iβ), a potent PP2A inhibitor through the phosphorylation of the PP2Ac tyrosine 307,[3] has been implicated in many cell processes such as DNA replication, chromatin remodeling and gene transcription,[4-5] differentiation,[6] migration[7] and cell-cycle regulation.[8] Moreover, numerous studies have shown that SET acts as an oncogene regulating important signaling pathways (reviewed in Westermarck and Hahn, 2008).[9] Consistent with its oncogenic role, *SET* is overexpressed in multiple solid tumors[10] and in chronic myeloid leukemia (CML), where its expression correlates with the oncogenic activity of BCR-ABL, leading to PP2A inhibition.[11] Moreover, in some acute leukemias, *SET* has been found fused to *CAN* or *HRX*, leading to the formation of fusion proteins that contribute to leukemogenesis by impairing the normal PP2A regulation.[12-13]

PP2A is a tumor suppressor that impairs cellular transformation by regulating a wide variety of signaling pathways (Reviewed in Mumby, 2007; and Westermarck and Hahn, 2008).[9,14] PP2A has been described as a potential therapeutic target in CML, Philadelphia-chromosome-positive acute lymphoblastic leukemia, and B-cell chronic lymphocytic leukemia.[11,15-16] In previous studies, we have described that overexpression of *SETBP1* in AML cells protects SET from protease cleavage, increasing the amount of SET protein, and forming a SETBP1-SET-PP2A complex that results in PP2A inhibition.[17] In addition, we observed that PP2A inactivation is a recurrent event in AML, and that restoration of PP2A activity by forskolin blocks proliferation, induces caspase-dependent apoptosis, and affects AKT and ERK1/2 activity.[18] Here, we show that SET deregulation is a recurrent event in AML in both cell lines and patient samples, where it is associated with a significant short overall survival. Analysis by western blot confirmed SET overexpression at protein level in AML patient samples. Ectopic expression of SET promotes cell proliferation and restores the reduced cell viability induced after PP2A overexpression. Moreover, analysis of the mechanisms responsible of SET deregulation showed that PP2A could regulate SET levels. The high recurrence of this alteration indicates that *SET* overexpression would represent a key inhibitory mechanism of PP2A in AML cells, and could discriminate a subgroup of patients susceptible for future therapies with PP2A activators.

Methods

EOL-1, HL-60, Kasumi-1, OCI-AML2, MOLM13, MV4-11, HEL, KG-1, K562, NOMO-1, UT-7, MUTZ-3, F-36P, TF-1 and HEK293 cell lines were maintained as previously described.[17-18] Cells were treated with the reagent forskolin (40 μM) (Calbiochem). For transfection experiments we used the Nucleofector System (Amaxa). Human *SET* cDNA was obtained by RT-PCR and cloned through EcoRI/BamHI sites into pEGFP-C2. *SET* insert was verified by sequencing. The study comprised bone marrow samples of 214 patients with AML at diagnosis. Although patients were treated with different schedules, all received regimes based on anthracycline and cytarabine as induction therapy. High dose cytarabine, and autologous or allogenic stem cell transplantation when possible, were used as consolidation therapy. All samples were taken anonymously. Total RNA was isolated using the RNeasy minikit (Qiagen). cDNA was synthesized with SuperScriptIII Reverse Transcriptase (Invitrogen). Quantification of *SET* was performed using a specific TaqMan Gene Expression Assay (Applied Biosystems) and GAPDH as internal control. Expression levels of miR-199b were determined using a specific TaqMan MicroRNA Assay (Applied Biosystems) and U6B as internal control. For quantification of miR-199b, total RNA was isolated using TRIzol Reagent (Invitrogen). Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C}{}_T$ method.[19] *SET* cut off was established as previously described (mean+5SD).[17] Protein extracts were isolated using TRIzol Reagent (Invitrogen) following manufacturer´s indications. Antibodies used were goat polyclonal anti-SET (Santa Cruz Biotechnology) and mouse monoclonal anti-βactin (Sigma). Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare). Cell proliferation (MTS assay) was measured as previously described.[18] Statistical analyses were performed using SPSS 15 for windows (SPSS Inc, Chicago Illinois). Overall survival (OS), disease-free survival (DFS) and event-free survival (EFS), defined as previously described,[17] were determined according to the Kaplan–Meier method. Survival comparisons were done with the Log-rank (LR) test if proportional hazard assumption was fulfilled, and Breslow (B) otherwise. Cox proportional hazards model was used to assess patient's outcome and was adjusted taking into consideration relevant parameters that included age, cytogenetic group, complete remission, and *SET* overexpression. $P<0.05$ was considered statistically significant. Bioinformatics analysis to identify the presence of hypothetical binding-sites for transcription factors in the *SET* proximal promoter was performed including a region containing 2,000 bps before the transcription start site, and was carried out using the MatInspector (www.genomatix.de) program. For Chromatin immunoprecipitation (ChIP), chromatin was fragmented with a Bioruptor (Diagenode). ChIP was carried out according to the manufacturer's protocol (High Cell ChIP kit, Diagenode) using anti-EVI1 (Cell Signaling) or an equal amount of IgG isotype as negative control (Cell Signaling) or anti-RNA Polymerase II, (Millipore) as positive control. Real time PCR (QRT-PCR) was used to amplify immunoprecipitated DNA using Power SybrGreen (Applied Biosystems). The amount of immunoprecipitated DNA in each experiment is represented as signal relative to the amount of input, and was calculated by QRT-

PCR using primers directed to promoter regions of *SET* and *PBX1* as positive control.[20] Primer sequences are included in Supplementary Table 1. Luciferase assays were done using the Dual luciferase system (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity.

Results and Discussion

The SET protein is a potent endogenous PP2A inhibitor that has been reported to be overexpressed in different human malignancies, including BCR-ABL positive leukemias.[11] In order to study if SET deregulation was a common event in AML we first analyzed by QRT-PCR the expression levels of *SET* in a panel of 13 AML BCR-ABL negative cell lines. Eight cell lines presented increased transcriptional levels of *SET*, and nine had SET overexpression at protein level (Supplementary Figure 1). However, some cell lines with normal or high SET mRNA levels have either overexpressed (HL-60 and Kasumi-1) or low SET protein (MUTZ-3 and TF-1). These observations would indicate that deregulation of SET is a common event in AML BCR-ABL negative cell lines, and that there is not always correlation between SET mRNA and protein.

To evaluate the clinical relevance of this alteration, we quantified the expression levels of *SET* by QRT-PCR in a series of 214 patients with AML at diagnosis (Table 1), correlated the results with cytogenetic and molecular markers, and studied the prognostic relevance of this aberration. *SET* was overexpressed in 28% cases (60/214), and was associated with cytogenetic prognostic groups ($p=0.004$), monosomy 7 ($p<0.01$), and *SETBP1* ($p<0.01$) and *EVI1* ($p=0.020$) overexpression (Table 2). Clinical follow-up data were available for 146 patients that received induction therapy (Supplementary Table 2), 72 men and 74 women, with a median age of 59 years (range 19-82). Median OS of the global cohort was 39.9 (95% CI 28.9-50.8 weeks) (Supplementary Figure 2). As expected, significant differences in OS according to age, cytogenetic group, and complete remission rate were found in this series ($p<0.01$) (Supplementary Figure 2). When we investigated the prognostic impact of *SET* in this cohort, we found that patients with *SET* overexpression had significantly worse OS ($p<0.01$) and EFS ($p<0.01$) (Figure 1 and Supplementary Figure 3). We found no differences in DFS ($p=0.229$) (Supplementary Figure 3). The prognostic impact of *SET* overexpression was significant in patients younger ($p=0.024$) and older ($p<0.01$) than 60 years (Supplementary Figure 4A). Interestingly, in the intermediate prognosis group, *SET* overexpression had prognostic impact in patients with normal karyotype (NK) ($p<0.01$), where it could define a subgroup of patients with poor outcome (Figure 1 and Supplementary Figure 4B). Almost half of adult AML is normal cytogenetically. Although the vast majority of NK patients can be individually characterized on the basis of distinct molecular markers, treatment of AML is still based on quite unspecific cytotoxic therapy, indicating the necessity to further investigate novel targeted therapies.[1,21] In addition, multivariate analysis demonstrated that *SET* overexpression is an unfavorable independent factor associated with OS in patients with AML ($p<0.01$). To further evaluate the importance of *SET* overexpression in AML, we analyzed SET at protein level in 16 of the AML cases of our series. Western blot showed a good correlation between SET mRNA and protein in 81% cases (13/16). We found increased levels of SET protein in 9 out of 16 cases (56.2%), although only 6 of these cases had SET overexpression by QRT-PCR (Figure 1). These results would suggest that SET overexpression is a common event in AML that could be underestimated by QRT-PCR, and have a higher prevalence at protein level.

Recent evidence has demonstrated that BCR-ABL induces SET upregulation, and the resulting PP2A inhibition, to fulfill its tumorigenic potential in CML (revised in Eichhorn et al., 2009).[22] Suppression of *SET* by shRNAs in BCR-ABL-positive cell lines resulted in increased PP2A activity and a reduction in leukemogenesis in vivo. On the contrary, *SET* overexpression conferred a proliferation advantage and completely rescued the cytokine-independent colony-forming ability of BCR-ABL cell lines ectopically expressing HA-PP2A.[11] Moreover, in a recent work our group reported that PP2A activation produces a decrease in cell proliferation in AML cells and that deregulation of *SET* could be one contributing mechanism to inhibit PP2A in AML.[17] Therefore, we assessed the effect of SET overexpression on cell growth in an AML BCR-ABL-negative cell model. MTS assay showed an increased proliferation in HEL cells transfected with SET compared with cells transfected with an empty vector (Figure 2A and Supplementary Figure 5). Similar results were observed in the KG-1 cell line (data not shown). Moreover, we observed that the decreased proliferation of HEL cells after the ectopic expression of PP2A was totally restored when cells were transfected with both SET and PP2A (Figure 2B). However, ectopic expression of SET only partially restored cell proliferation after treatment with the PP2A activator forskolin (Figure 2C), suggesting an additional toxicity of this drug on AML cells, independent of PP2A activation. Altogether, these results demonstrate that SET overexpression promotes cell proliferation and restores the effect of PP2A overexpression on cell growth in AML cells.

Neviani et al (2005) showed that enhanced BCR-ABL activity is the mechanism that induces SET overexpression in BCR-ABL-positive cells. So, we next investigated the molecular mechanisms involved in *SET* deregulation in AML. In a recent report, *SET* has been described as target of miR-199b in human choriocarcinoma.[23] In order to test if an altered expression of miR-199b could be deregulating *SET* in AML cells, we analyzed the expression levels of miR-199b in 13 AML BCR-ABL-negative cell lines, observing that only EOL-1, UT-7 and TF-1 presented downregulation of miR-199b (Supplementary Figure 6). However, neither UT-7 nor TF-1 have *SET* overexpression (Supplementary Figure 1), and only in EOL-1 decrease levels of miR-199b could explain, at least in part, the overexpression of *SET*. These results indicate that downregulation of miR-199b is not the key mechanism responsible for the *SET* overexpression in AML. Transformed cells present a wide variety of mechanisms to inactivate PP2A, including alterations in structural PP2A subunits, overexpression of specific endogenous inhibitors, and suppression of regulatory subunits.[9,14,22] We have previously described that several of these mechanisms could be acting in AML patients at the same time.[18] Therefore, we hypothesized that PP2A inactivation could regulate SET in AML. We treated HEL cells with the PP2A activator forskolin, observing a decrease in the expression levels of SET compared with vehicle treated cells (Figure 2D). These results would indicate that activation of PP2A leads to a reduction of *SET* levels, and suggest that loss of this control by the inhibited status of PP2A could contribute to deregulate *SET* in AML cells. In order to analyze other molecular events that could deregulate *SET* in AML, we performed a bioinformatic analysis of the proximal promoter of the *SET* gene. We identified several hypothetical binding-sites for transcription factors previously implicated in AML such as HMG-1, AP-1, SP1, E2F1, GATA1, and EVI1. In our series of AML patients, *SET* overexpression was associated with overexpression

of *EVI1*, suggesting that this transcription factor could be a candidate to regulate *SET*. ChIP showed that EVI1 binds the *SET* promoter (Supplementary Figure 7A). However, we did not detect differences in luciferase activities after co-transfection with pGL3-SETPromoter and pCMV6-EVI1 (Supplementary Figure 7B). These results would discard the transcription factor EVI1 as a direct regulator of *SET*, although could suggest that EVI1 regulates *SET* indirectly, through other transcriptional mechanisms.

In summary, we show that deregulation of *SET* is a recurrent molecular event in AML, that promotes cell proliferation and restores the reduced cell viability induced after PP2A overexpression. In addition, analysis of a series of patients indicates that *SET* overexpression is associated with a significant poor overall survival in AML. Investigation about the molecular mechanisms responsible of *SET* deregulation showed that EVI1 and PP2A could regulate the transcriptional levels of *SET*. Increased understanding of the biology of AML is leading to targeted approaches to develop more effective treatments for this disease. In this line of thinking, *SET* overexpression differentiates a subgroup of patients with poor prognosis that could be treated with PP2A activators in future clinical trials.

Acknowledgements: This work was supported by Ministerio Educación y Ciencia (SAF2005/06425 and AP2006-03038), Ministerio Ciencia e Innovación (PI081687), Gobierno de Navarra (14/2008), ISCIII-RTICC (RD06/0020/0078), and FIMA y UTE (Spain).

Authorship Contributions: IC, LGO, CC and MGS performed research; IC and MDO designed research and wrote the paper.

Conflict of interest disclosure: No potential conflicts of interest were disclosed.

Tables

Table 1. Clinical and molecular characteristics of a series of 214 patients with AML at diagnosis.

		No. (%)
Sex		
	Male	112 (53.8)
	Female	96 (46.2)
	No data	6
Age		
	<60 years	99 (48.5)
	>60 years	105 (51.5)
	No data	10
Complete Remission		
	No	51 (36.7)
	Yes	88 (63.3)
	No data	75
Diagnosis		
	AML-M0	19 (8.8)
	AML-M1	42 (19.6)
	AML-M2	47 (22)
	AML-M3	7 (3.3)
	AML-M4	35 (16.3)
	AML-M5	37 (17.3)
	AML-M6	14 (6.6)
	AML-NOS	13 (6.1)
Secondary AML (sAML)		
	No	141 (81)
	Yes	33 (19)
	No data	40
Cytogenetic group		
	good	29 (13.6)
	intermediate	108 (50.4)
	poor	77 (36)
SET overexpression		
	No	154 (72)
	Yes	60 (28)
SETBP1 overexpression		
	No	139 (72.4)
	Yes	53 (27.6)
	No data	22
WT1 overexpression		
	No	23 (15.8)
	Yes	122 (84.2)
	No data	69
EVI1 overexpression		
	No	142 (77.6)
	Yes	41 (22.4)
	No data	31
MDS1EVI1 overexpression		
	No	174 (91.6)
	Yes	16 (8.4)
	No data	24
FLT3-ITD		
	No	116 (80)
	Yes	29 (20)
	No data	69
NPM1 mutated		
	No	22 (40.7)
	Yes	32 (59.3)
	No data	160

Table 2. Association between *SET* overexpression and clinical and genetic parameters in 214 patients with AML at diagnosis.

		No. Cases	No. SET- (%)	No. SET+ (%)	P
SET		214	154 (72)	60 (28)	
Sex		208	149	59	0.319
Male		112	77 (68.7)	35 (31.3)	
Female		96	72 (75)	24 (25)	
Age		204	146	58	0.505
<60 years		99	73 (73.7)	26 (26.3)	
≥ 60 years		105	73 (69.5)	32 (31.5)	
CR		128	180	50	0.571
No		51	36 (70.6)	15 (29.4)	
Yes		88	66 (75)	22 (25)	
sAML		174	129	45	0.126
No		141	108 (76.6)	33 (23.4)	
Yes		33	21 (63.6)	12 (36.4)	
Prognostic group		214	154	60	**0.004**
good		29	22 (76)	7 (24)	
intermediate		108	87 (80.6)	21 (19.4)	
poor		77	45 (58.4)	32 (41.6)	
Cytogenetic group					
normal karyotype	yes	72	54 (56.2)	18 (43.8)	0.520
	no	137	97 (70.8)	40 (29.2)	
trisomy 8	yes	17	14 (82.3)	3 (17.6)	0.297
	no	179	126 (70.4)	53 (29.6)	
chromosome 18 aberrations	yes	8	5 (62.5)	3 (37.5)	0.527
	no	187	136 (72.7)	51 (27.3)	
monosomy 7	yes	31	16 (51.6)	15 (48.4)	**0.006**
	no	166	126 (75.8)	40 (24.2)	
der(7q)	yes	38	22 (57.8)	16 (42.2)	**0.030**
	no	166	123 (74)	43 (26)	
SETBP1 overexpression		192	137	55	**0.000**
No		139	109 (78.4)	30 (21.6)	
Yes		53	28 (52.8)	25 (47.2)	
WT1 overexpression		145	113	32	0.612
No		23	17 (86.7)	6 (13.6)	
Yes		122	96 (77.4)	26 (22.6)	
EVI1 overexpression		183	137	46	**0.020**
No		142	112 (78.8)	30 (21.2)	
Yes		41	25 (61)	16 (39)	
MDS1EVI1 overexpression		190	144	46	0.492
No		174	133 (76.4)	41 (23.6)	
Yes		16	11 (68.7)	5 (31.3)	
FLT3-ITD		145	111	34	0.972
No		116	89 (76.7)	27 (23.3)	
Yes		29	22 (75.8)	7 (24.2)	
NPM1 mutated and *FLT3* wt		54	46	11	0.341
No		17	15 (88.2)	2 (11.8)	
Yes		21	16 (76.2)	5 (23.8)	

Figures

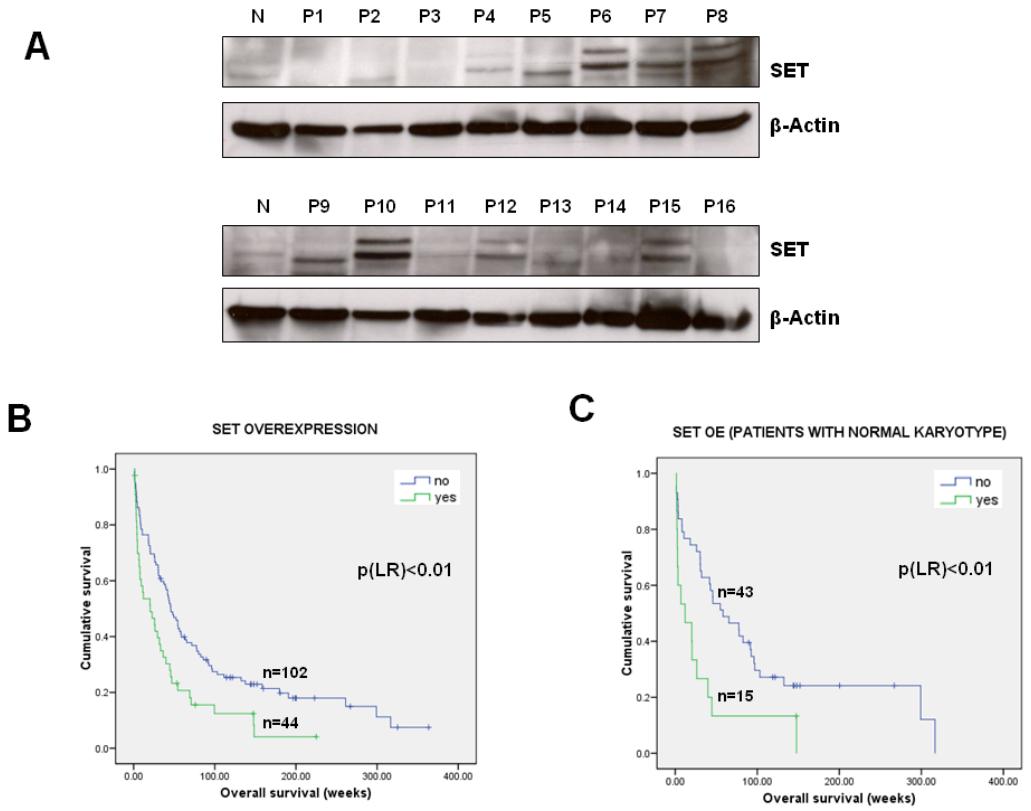


Figure 1. SET is deregulated in samples of patients with AML and confers poor outcome. (A) Western blot analysis showing SET expression levels in 16 AML patients at diagnosis; (B) Kaplan-Meier analyses of overall survival for *SET* overexpression in a series of 146 patients with AML and clinical follow-up data available who received induction therapy; (C) Kaplan-Meier analyses of overall survival for *SET* overexpression in the subgroup of patients with normal karyotype.

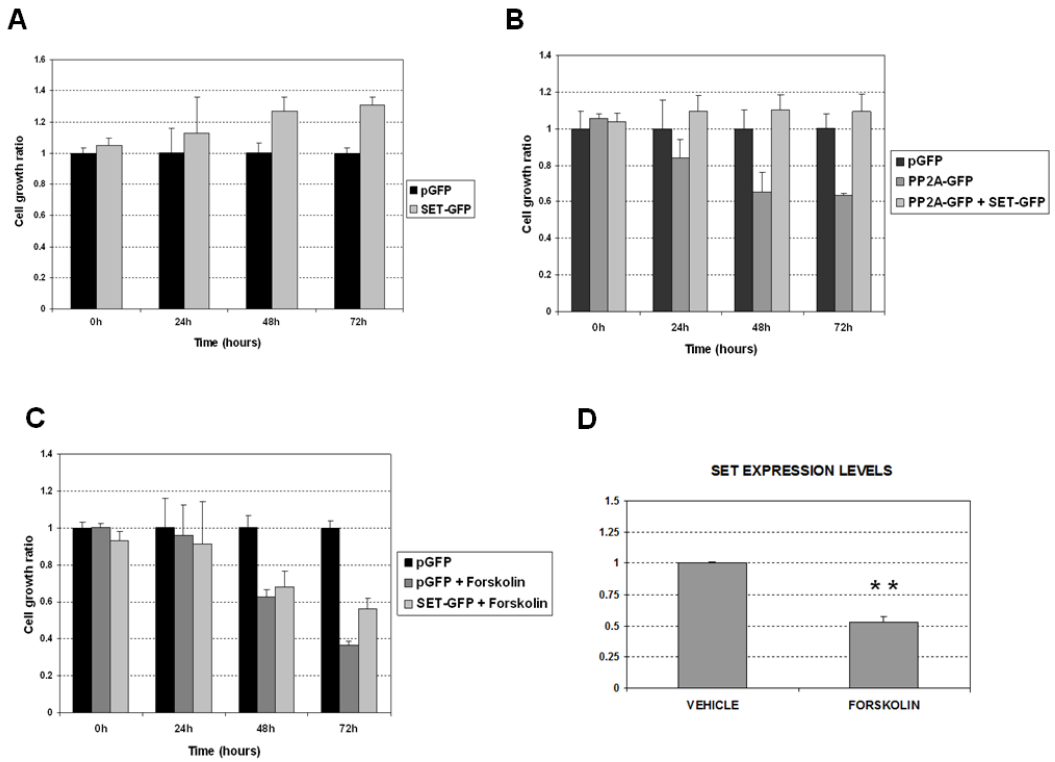


Figure 2. SET restores cell proliferation in ectopically PP2A-expressing AML cells, and its expression levels are dependent on PP2A activation status. (A) MTS assay showing proliferation in HEL cells transfected with SET compared to cells transfected with an empty vector; (B) MTS assay showing proliferation in HEL cells transfected with PP2A alone or in combination with SET or with an empty vector; (C) MTS assay showing the effect of SET in cells treated with the PP2A activator forskolin; (D) Analysis by real-time PCR of *SET* expression levels after in vehicle and forskolin treated HEL cells; ***P*<0.01**.**

References

1. Fathi AT, Grant S, Karp JE. Exploiting cellular pathways to develop new treatment strategies for AML. *Cancer Treat Rev*. 2010;36(2):142-150.

2. Stone RM, O'Donnell MR, Sekeres MA. Acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*. 2004:98-117.

3. Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem*. 1996;271(19):11059-11062.

4. Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell*. 2001;104(1):119-130.

5. Kutney SN, Hong R, Macfarlan T, Chakravarti D. A signaling role of histone-binding proteins and INHAT subunits pp32 and Set/TAF-Ibeta in integrating chromatin hypoacetylation and transcriptional repression. *J Biol Chem*. 2004;279(29):30850-30855.

6. Kandilci A, Mientjes E, Grosveld G. Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937. *Leukemia*. 2004;18(2):337-340.

7. ten Klooster JP, Leeuwen I, Scheres N, Anthony EC, Hordijk PL. Rac1-induced cell migration requires membrane recruitment of the nuclear oncogene SET. *EMBO J*. 2007;26(2):336-345.

8. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, et al. The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. *J Biol Chem*. 2003;278(2):1158-1164.

9. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med*. 2008;14(4):152-160.

10. Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. *J Biol Chem*. 2002;277(28):25026-25031.

11. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR-ABL-regulated SET protein. *Cancer Cell*. 2005;8(5):355-368.

12. von Lindern M, van Baal S, Wiegant J, Raap A, Hagemeijer A, Grosveld G. Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. *Mol Cell Biol*. 1992;12(8):3346-3355.

13. Adler HT, Nallaseth FS, Walter G, Tkachuk DC. HRX leukemic fusion proteins form a heterocomplex with the leukemia-associated protein SET and protein phosphatase 2A. *J Biol Chem*. 1997;272(45):28407-28414.

14. Mumby M. PP2A: unveiling a reluctant tumor suppressor. *Cell*. 2007;130(1):21-24.

15. Neviani P, Santhanam R, Oaks JJ, et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest*. 2007;117(9):2408-2421.

16. Liu Q, Zhao X, Frissora F, et al. FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. *Blood*. 2008;111(1):275-284.

17. Cristobal I, Blanco FJ, Garcia-Orti L, et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood*. 2010;115(3):615-625.

18. Cristobal I, Garcia-Orti L, Cirauqui C, Alonso MM, Calasanz MJ, Odero MD. PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect. *Leukemia*. 2011.

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.

20. Shimabe M, Goyama S, Watanabe-Okochi N, et al. Pbx1 is a downstream target of Evi-1 in hematopoietic stem/progenitors and leukemic cells. *Oncogene*. 2009;28(49):4364-4374.

21. Zaidi SZ, Owaidah T, Al Sharif F, Ahmed SY, Chaudhri N, Aljurf M. The challenge of risk stratification in acute myeloid leukemia with normal karyotype. *Hematol Oncol Stem Cell Ther*. 2008;1(3):141-158.

22. Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta*. 2009;1795(1):1-15.

23. Chao A, Tsai CL, Wei PC, et al. Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. *Cancer Lett*. 2010;291(1):99-107.

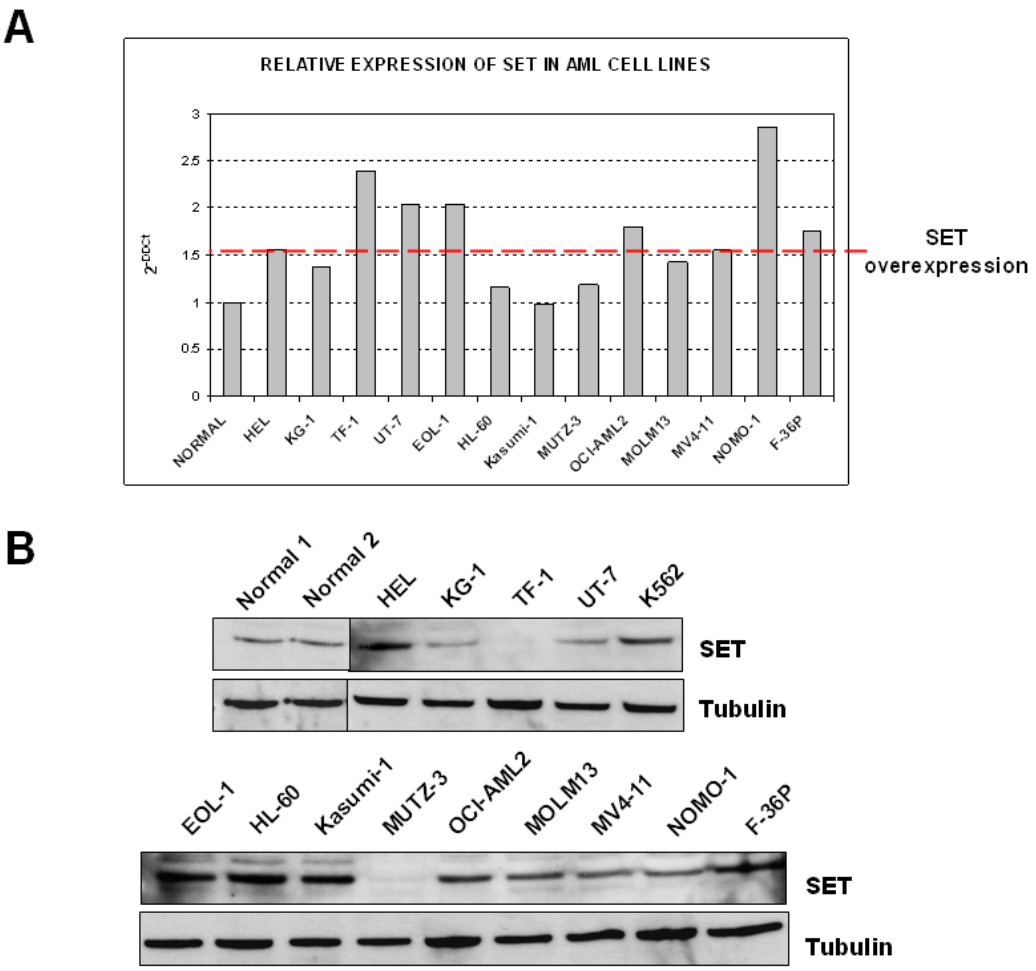
Supplementary Information

Supplementary Table 1. Primer sequences used for ChIP analysis.

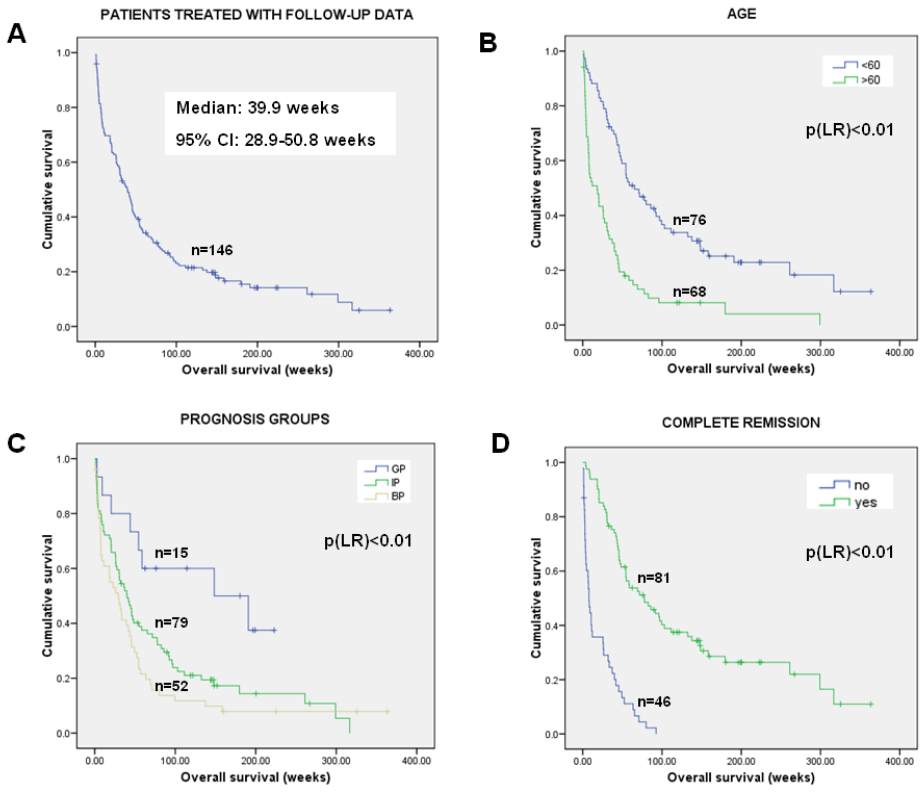
Name	Sequence
SET-forward	GTACCTCCCTGCCTTCACAG
SET-reverse	CCTTGAGCAAGTCACCCTTC
PBX1-forward	CAAAGGAGACCGGACTGAAA
PBX1-reverse	GGAAGGGTGTTGACTCCAAA

Supplementary Table 2. Clinical and molecular characteristics of a series of 146 patients with AML at diagnosis and clinical follow-up data who received induction therapy.

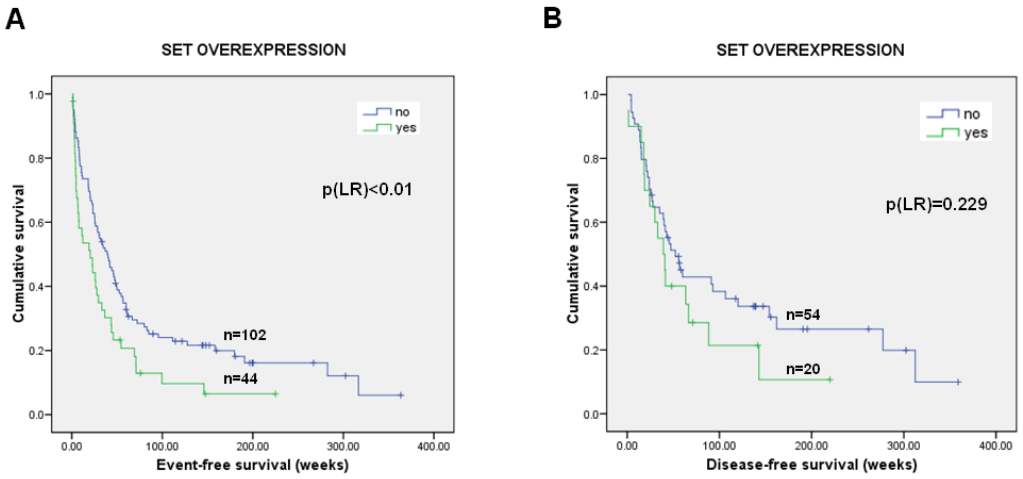
		No. (%)	
Sex			
	Male	72	(49.3)
	Female	74	(50.7)
Age			
	<60 years	76	(52.8)
	>60 years	68	(47.2)
	No data	2	
Complete Remission			
	No	46	(35.7)
	Yes	81	(64.3)
	No data	19	
Diagnosis			
	AML-M0	12	(8.2)
	AML-M1	36	(24.7)
	AML-M2	36	(24.7)
	AML-M4	22	(15)
	AML-M5	23	(15.8)
	AML-M6	10	(6.8)
	AML-NOS	7	(4.8)
Secondary AML (sAML)			
	No	98	(67)
	Yes	16	(33)
	No data	32	
Cytogenetic group			
	good	15	(10.2)
	intermediate	79	(54.2)
	poor	52	(35.6)
SET overexpression			
	No	102	(69.8)
	Yes	44	(30.2)
SETBP1 overexpression			
	No	101	(73.7)
	Yes	36	(26.3)
	No data	9	
WT1 overexpression			
	No	16	(16.2)
	Yes	83	(83.8)
	No data	47	
EVI1 overexpression			
	No	94	(72.8)
	Yes	35	(27.2)
	No data	17	
MDS1EVI1 overexpression			
	No	120	(91.6)
	Yes	11	(8.4)
	No data	15	
FLT3-ITD			
	No	77	(77)
	Yes	23	(22)
	No data	46	
NPM1 mutated			
	No	19	(38.8)
	Yes	30	(61.2)
	No data	97	



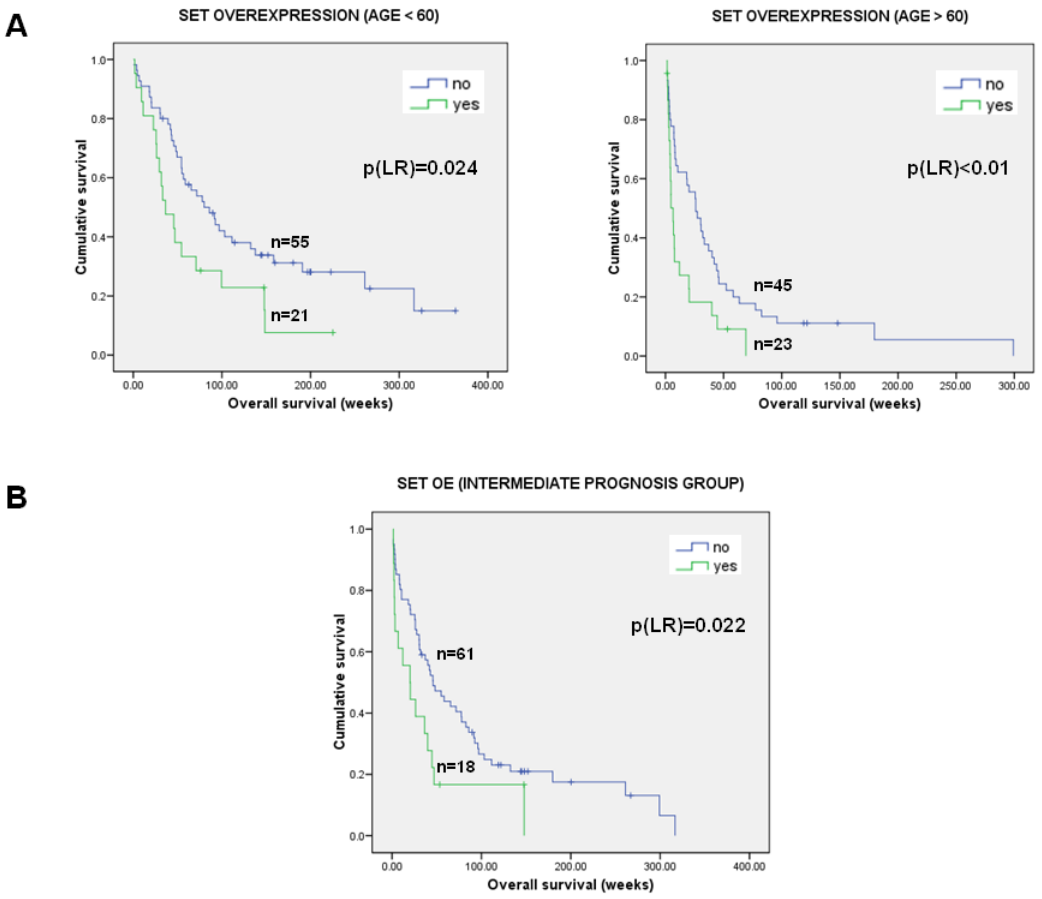
Supplementary Figure 1. SET is deregulated in AML cell lines. (A) Analysis by real-time PCR of *SET* expression levels in 13 AML cell lines; (B) Western blot analysis showing SET expression in 13 AML cell lines. Protein extract of the CML cell line K562 was included as positive control of SET overexpression.[11]



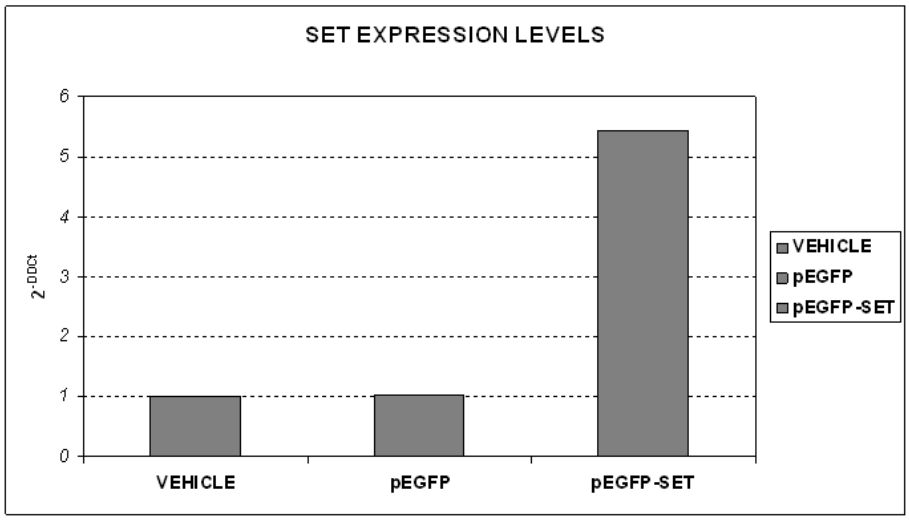
Supplementary Figure 2. Kaplan-Meier analyses of overall survival in the cohort of 146 patients treated with clinical follow-up data available. (A) Survival graph of the global cohort; An inferior outcome was observed in patients older than 60 years (B), patients included in the intermediate and poor prognosis groups (C), and patients who did not achieve CR (D).



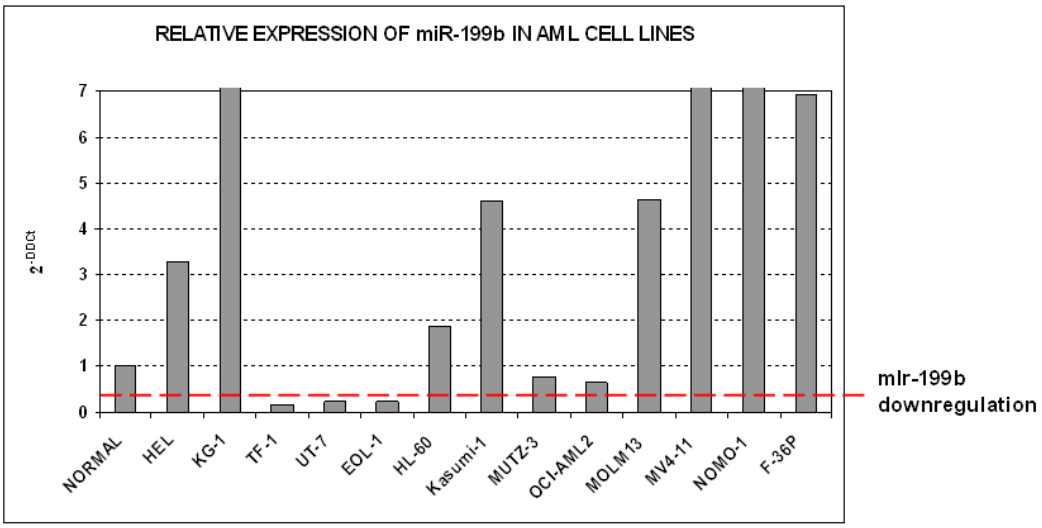
Supplementary Figure 3. Kaplan-Meier analysis of disease-free survival for *SET* overexpression in a series of 146 patients with AML and clinical follow-up data available who received induction therapy. (A) Event-free survival; (B) disease-free survival.



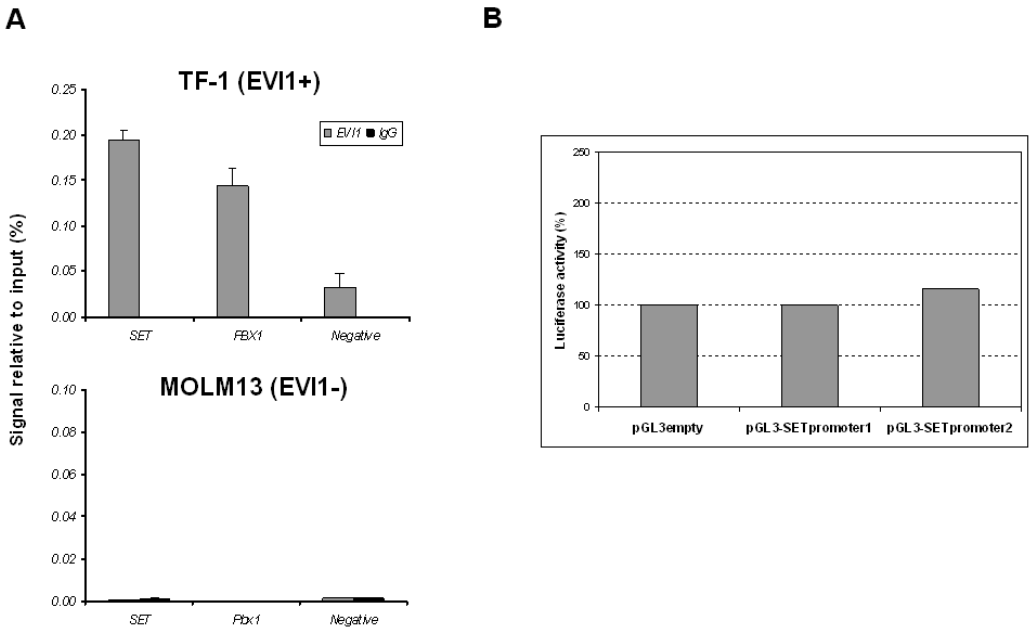
Supplementary Figure 4. Kaplan-Meier analyses of overall survival for *SET* overexpression. (A) Series of age-stratified 146 treated patients with AML, and clinical follow-up data available; (B) Series of patients included in the intermediate and prognosis group.



Supplementary Figure 5. Analysis by real-time PCR of *SET* expression levels after transfection in HEL cells.



Supplementary Figure 6. Analysis by real-time PCR of the expression levels of miR-199b in 13 AML BCR-ABL negative cell lines.



Supplementary Figure 7. ChIP and Luciferase analyses on the SET proximal promoter. (A) Specific DNA binding of EVI1 to *SET* promoter was detected by ChIP. Real time PCR was performed on fragmented chromatin precipitated by anti-EVI1 antibody (grey bars) or Normal Rabbit IgG (black bars) from an EVI1 positive (EVI1+) TF1 cell line and the EVI1 negative (EVI1-) MOLM13 cell line. Primers were designed to amplify the promoter region of *SET*. The EVI1 positive target gene *PBX1* was used as positive control. Results were expressed as percentage of input; (B) Luciferase reporter assay in HEK293 cells co-transfected with pGL3-SETpromoter and pCMV6-EVI1 or an empty vector.

DISCUSIÓN GENERAL

El análisis mediante arrays de SNPs y mRNAs junto con el perfil de expression de miRNAs ha permitido la identificación de NF1 como diana del miR-370 en leukemia mieloide aguda

Los miRNAs son pequeños RNAs (21-25 nucleótidos) endógenos que regulan la expresión de otros genes uniéndose a secuencias específicas de regiones 3´UTR del RNA mensajero diana y reprimiendo su traducción en función de la complementariedad entre el miRNA y el RNA mensajero. Su expresión está muy regulada a nivel temporal y espacial, dependiendo del linaje celular y de su grado de diferenciación. Se ha demostrado que algunos miRNAs presentan una expresión aberrante en determinados tumores, pudiendo actuar como oncogenes o genes supresores de tumores, regulando diversos procesos biológicos a través de cientos de genes diana, lo que sugiriere una influencia potencial en casi todas las vías de señalización.

Se han descrito como mecanismos de desregulación de miRNAs en cáncer la presencia de mutaciones, translocaciones, alteraciones epigenéticas, y también desregulaciones en el mecanismo de procesamiento de los miRNAs. Sin embargo, las alteraciones del número de copias (CNV) representan un mecanismo alternativo poco estudiado hasta el momento. En este sentido, nuestro grupo ha analizado la importancia de este mecanismo en leucemia mieloide aguda (LMA) mediante un estudio que integra datos procedentes de arrays de SNPs, arrays de expresión, y el perfil de expresión de 250 miRNAs maduros. De este modo, se han identificado 19 miRNAs cuya expresión correlacionaba significativamente con la presencia de CNVs en las regiones genómicas en que esos miRNAs están localizados. Estos datos confirman que las CNVs son uno de los mecanismos responsables de la alteración de expresión de miRNAs en LMA.

La asociación entre alteraciones genéticas específicas de LMA y miRNAs ha sido investigada recientemente en cinco trabajos (Marcucci et al., 2008; Garzon et al., 2008; Garzon et al., 2008; Dixon-McIver et al., 2008; Li et al., 2008) y revisada en Marcucci et al., (2011). Estos trabajos demuestran que algunos subtipos citogenéticos y moleculares de LMA pueden clasificarse de acuerdo a su patrón de expresión de miRNAs.

En nuestro estudio, el análisis conjunto de los datos de arrays de expresión y la identificación bioinformática de las posibles dianas génicas de los 19 miRNAs cuya expresión estaba alterada por la presencia de CNVs, nos permitió identificar las posibles dianas de 9 de estos 19 miRNAs. Nuestra hipótesis era que la presencia de esas CNVs que alteraban la expresión de miRNAs estaría, en última instancia, desregulando la expresión de los genes regulados por dichos miRNAs. Comenzamos el estudio de los genes *NF1* y c-*CBL*, ya implicados en cáncer, que además eran los genes candidatos para 5 de los miRNAs en estudio.

NF1 es un gen supresor tumoral que regula negativamente la vía de señalización RAS (Mullaly et al., 2010). Se ha decrito su desregulación y la presencia de mutaciones en casos de leucemias infantiles y en leucemia mielomonocítica juvenil (Loh et al., 2010). Por otra parte, se ha comprobado que la deficiencia de *NF1* coopera con la proteína oncogénica K-RAS en la inducción de leucemia aguda en ratones (Cutts et al., 2009). Un estudio reciente muestra que el 7% de los casos con LMA (7/95) tienen una expresión nula de *NF1* debido a alteraciones genómicas. Además, la ausencia de expresión de *NF1* se asoció con un

incremento de actividad Ras. Este hecho podría ser clínicamente relevante al permitir discriminar a un subgrupo de pacientes con LMA susceptibles de recibir terapias dirigidas hacia Ras, y por el hecho de que los blastos de este tipo de pacientes presentan una mayor sensibilidad a la citarabina (Parkin et al., 2010). En nuestro estudio, demostramos por primera vez que una de las causas de la disminución de la expresión de *NF1* puede ser la acción del miR-370. Hemos observado que el miR-370 tiene como diana directa a *NF1*, confirmando las observaciones realizadas a nivel proteico en las que se observaba una disminución de los niveles de NF1 tras la expresión ectópica del miR-370. Además, el análisis en una serie de 31 pacientes con LMA al diagnóstico mostró niveles reducidos de *NF1* en 19 de los 31 casos, de los cuales 9 presentaban sobreexpresión del miR-370. Estos resultados indicarían que la expresión reducida de *NF1* es un evento recurrente en LMA, y que la sobreexpresión del miR-370 parece ser una causa importante de la menor expresión de *NF1*. Sin embargo, 10 de los 19 casos con *NF1* reducido no presentaron sobreexpresión del miR-370, lo que indica que deben actuar también otros mecanismos para regular la expresión de *NF1*. Por lo tanto, nuestros resultados proporcionan un nuevo mecanismo de regulación del supresor tumoral *NF1* en leucemia.

Por otra parte, hemos comprobado que la sobreexpresión del miR-379 provoca una disminución en los niveles de c-CBL; sin embargo, los ensayos de luciferasa mostraron que no se trataba de una diana directa. Por tanto, miR-379 parece regular los niveles de c-CBL de forma indirecta, probablemente controlando los niveles de expresión de algún factor de transcripción implicado en la regulación de c-*CBL*. La familia *CBL* incluye un grupo de ubiquitina-ligasas, para las que se han encontrado mutaciones en una gran variedad de tumores, incluyendo la LMA (Kales et al., 2010). Además, se ha visto que CBL puede disminuir los niveles de FLT3, un receptor tirosina quinasa frecuentemente activado en LMA (Sargin et al., 2007). Nuestros resultados indican que la desregulación del miR-379 podría representar un mecanismo alternativo para alterar los niveles de expresión de c-*CBL*, aunque hacen falta más estudios para determinar el modo por el que el miR-379 actúa en c-*CBL*.

En resumen, en este trabajo se ha llevado a cabo un estudio que integra datos procedentes tanto de arrays de SNPs y expresión, como de los perfiles de miRNAs de 16 líneas mieloides. Así, mediante este método se pudieron identificar 19 miRNAs cuya expresión correlacionaba significativamente con la presencia de CNVs en las regiones genómicas en las cuales estos miRNAs se encuentran localizados. Además, el estudio de los niveles de expresión de los genes diana predichos mediante análisis bioinformático, ha permitido identificar a *NF1* como diana directa del miR-370, y determinar que el miR-379 es capaz de regular de forma negativa e indirecta los niveles de c-CBL. Por tanto, con los resultados aportados en este trabajo queda de manifiesto que la presencia de CNVs en regiones genómicas que incluyen miRNAs, representa un mecanismo alternativo de desregulación de la expresión génica en LMA.

Por otra parte, la caracterización de las líneas celulares mieloides ha permitido utilizarlas como modelos celulares en los siguientes objetivos que nos planteamos en este estudio.

PP2A está frecuentemente inhibido en leucemia mieloide aguda y su activación mediante forskolina muestra un efecto antileucémico

La proteína fosfatasa PP2A funciona como un supresor tumoral que inhibe la transformación celular regulando la actividad de proteínas de señalización críticas para el comportamiento celular maligno. La inhibición de PP2A juega un papel clave en leucemias BCR/ABL positivas como LMC y LLA (Neviani et al., 2005; Neviani et al., 2007), en precursores mieloides c-KIT positivos (Roberts et al., 2010), y también en LLC (Liu et al., 2008). Además, la activación de PP2A tanto por forskolina como por FTY720 parece tener efectos terapéuticos prometedores en estas enfermedades. Se ha descrito la desregulación de muchas quinasas en LMA pero, sin embargo, el papel de las fosfatasas en el proceso de transformación celular ha sido poco estudiado (Westermarck et al., 2008), y sólo dos trabajos analizan el papel de PP2A en LMA (Gallay et al., 2009; Roberts et al., 2010). Nuestros resultados tanto con líneas celulares como con muestras de pacientes con LMA confirman que la inhibición de PP2A es un evento recurrente que juega un papel importante en el desarrollo de LMA, ya que la activación farmacológica de PP2A in vitro es capaz de revertir algunas de las características de la célula leucémica.

Por otra parte, el estudio de PP2A a nivel proteico en 37 muestras de pacientes con LMA al diagnóstico, mostró una inhibición de PP2A en el 78% de los casos. En 6 de los 8 casos sin hiperfosforilación en la tirosina 307 de la subunidad catalítica de PP2A (PP2Ac) se encontró una expresión reducida de la misma. Esta observación es consistente con la menor expresión de PP2Ac observada en las líneas celulares Kasumi-1 y MUTZ-3, e indicaría que la reducción de expresión de PP2Ac podría ser otro mecanismo involucrado en la inhibición de la actividad PP2A en LMA. Por otra parte, también observamos que la activación de PP2A por forskolina presenta un efecto antileucémico aditivo con el tratamiento con Idarrubicina y Ara-c en las líneas celulares HEL y KG-1, lo que sugeriría una alternativa terapéutica basada en la combinación de la activación de PP2A con la terapia estándar de inducción empleada hoy en día. Además, se ha descrito que las concentraciones de forskolina empleadas en este estudio no afectan a la viabilidad de las células normales de médula ósea (Neviani et al., 2005). Otro hecho interesante fue que la forskolina afectó al estado de activación de AKT y ERK1/2 de una forma sensible al ácido okadaico, indicando que su acción es dependiente de la activación de PP2A. Esto concuerda con el hecho de que varias dianas de PP2A se han visto desreguladas en LMA y que algunas como AKT se han asociado con peor pronóstico en LMA (Gallay et al., 2009).

Los resultados obtenidos en este trabajo confirman que la inactivación de PP2A podría ser uno de los sucesos que contribuyen a estas alteraciones, ya que PP2A juega un papel central en la regulación de un gran número de vías de señalización esenciales, cuya desregulación está asociada con el proceso tumoral (Eichhorn et al., 2009). Hallazgos recientes han aportado evidencias de que la supresión de la actividad PP2A coopera con otras alteraciones en el proceso de transformación de muchos tipos celulares (Mumby, 2007; Sablina et al., 2007; Junttila et al., 2008). En nuestra serie encontramos que el 34% de los pacientes con inactivación de PP2A (10/29) tenían *FLT3*-ITD y/o mutaciones en *NPM1*. Resulta de interés que la activación constitutiva de *FLT3* en los casos con *FLT3*-ITD también lleva a la activación tanto de AKT como de ERK1/2 (Kornblau et al., 2010), lo que sugiere

que en algunos pacientes podrían colaborar la inactivación de PP2A con la presencia de *FLT3*-ITD en el desarrollo de LMA.

PP2A puede ser inhibido por el antígeno tumoral pequeño (ST) de algunos virus, por la sobreexpresión de inhibidores endógenos de PP2A, por la presencia de mutaciones inactivadoras que afectan a las subunidades estructurales, o por una expresión reducida de subunidades de PP2A tanto estructurales como reguladoras (Westermarck et al., 2008; Eichhorn et al., 2009). Por otra parte, la activación constitutiva de *JAK2* podría también contribuir independientemente a la inactivación de PP2A (Samanta et al., 2009; Yokoyama et al., 2001). Los resultados aportados por este trabajo muestran que los mecanismos de inactivación de PP2A en LMA podrían ser la sobreexpresión de los inhibidores *CIP2A* (Junttila et al., 2007) y *SET* (Li et al., 1996), así como la expresión reducida de ciertas subunidades de PP2A, lo cual sugiere que sería la disfunción de varios de los complejos PP2A los que podrían contribuir a la transformación celular.

La sobreexpresión de *CIP2A* o *SET* podrían explicar el mecanismo de inactivación de PP2A en el 58% de los casos (17/29). *SET* está sobreexpresado en varios tipos de cáncer (Cervoni et al., 2002), y se ha visto fusionado con *NUP214*/*CAN* en un paciente con LMA (Rosati et al., 2007). Además, Neviani et al. (2005) demostraron que la inactivación de PP2A en LMC es debida a la expresión elevada de SET, inducida por el incremento de actividad BCR/ABL, de modo que SET aumenta progresivamente de niveles a medida que tiene lugar la transformación a crisis blástica. De hecho, tanto el tratamiento con imatinib como la supresión de *SET* son capaces de restaurar los niveles normales de actividad PP2A (Neviani et al., 2005). Nuestros resultados muestran que el aumento de expresión de SET también conduce a una inactivación de PP2A en LMA, de forma independiente de la inducción por BCR/ABL. La mutación activadora *JAK2*-V617F fue detectada en una de las muestras con hiperfosforilación de PP2A pero también en tres muestras sin hiperfosforilación de PP2A, lo cual indica que JAK2 probablemente necesite de alguna alteración adicional para inducir la inactivación de PP2A.

Debido a que en 12 casos el mecanismo de inhibición de PP2A no pudo ser determinado, y uno de los casos tenía una delección del(11)(q13q23), una región donde se encuentra localizada la subunidad estructural *PPP2R1B* (11q23) y la reguladora *PPP2R5B* (11q13), pensamos que la expresión alterada de alguna de sus subunidades podría afectar a la actividad PP2A, como ya previamente había sido descrito (Eichhorn et al., 2009). El análisis mediante arrays de SNPs y de expresión en 16 líneas celulares mieloides demostró la existencia de alteraciones afectando a subunidades de PP2A, que podrían estar jugando un importante papel en la inactivación de PP2A observada en LMA. Así, encontramos una menor expresión de *PPP2R5B* y *PPP2R5C* tanto en líneas celulares de LMA como de LMC. La subunidad reguladora PPP2R5B funciona como un supresor tumoral que regula negativamente a la quinasa Pim-1, la cual se sabe que incrementa la capacidad de c-Myc de inducir linfomas (Ma et al., 2007). Por otra parte, se ha descrito que la supresión de *PPP2R5C* contribuye a la transformación de células humanas (Chen et al., 2004). Nuestros datos en líneas celulares y en muestras de pacientes sugieren que la pérdida de *PPP2R5B* y *PPP2R5C* podría ser importante

en el desarrollo de LMA por su contribución a la desregulación de una función PP2A correcta. También observamos que es común la expresión disminuída de la subunidad estructural *PPP2R1B* en LMA, para la que previamente ya se han descrito mutaciones en varios tipos de cáncer (Ruediger et al., 2001; Xu et al., 2006; Cho et al., 2007). Además, el trabajo de Sablina et al. (2007) proporcionó evidencias de que estas mutaciones contribuyen a la transformación celular. Sin embargo, son necesarios más estudios para clarificar la importancia real de la disminución de la expresión de estas subunidades de PP2A en LMA.

En nuestro estudio, hemos visto que la inhibición de PP2A es un evento recurrente que juega un papel importante en el desarrollo de la LMA. Además, la inactivación de PP2A ocurre a través de diferentes mecanismos entre los que se encuentran la sobreexpresión de sus inhibidores endógenos o la menor expresión de ciertas subunidades del complejo PP2A. Por otra parte, hemos demostrado que la activación de PP2A por el tratamiento con forskolina reduce la proliferación celular, induce una apoptosis dependiente de caspasas, y modifica el estado de activación de dianas de PP2A como AKT y ERK1/2. Los resultados obtenidos señalan además que la activación de PP2A tiene un potencial terapéutico en combinación con drogas usadas en terapia estándar de indución como Idarrubicina y Ara-c.

La sobreexpresión de *SETBP1* constituye un nuevo mecanismo que contribuye al desarrollo de leucemia mieloide aguda y un marcador de mal pronóstico en pacientes con edad avanzada

En este estudio hemos descrito un nuevo mecanismo de transformación en LMA. Nuestro trabajo comenzó con el análisis de un paciente con LMA y una translocación t(12;18)(p13;q12) en la que estaba implicado el gen *ETV6*. El hecho de no encontrar ninguna fusión con significado funcional nos llevó a pensar que el hecho relevante podría ser la sobreexpresión de algún gen cercano al punto de rotura. Identificamos que el gen *SETBP1,* localizado cerca del punto de rotura, estaba sobreexpresado tanto en la muestra del paciente al diagnóstico, como en muestras post-tratamiento. Teniendo en cuenta que la activación de la expresión de *SETBP1* por inserción retroviral en células progenitoras hematopoyéticas se había descrito que confería una ventaja proliferativa a esas células (Ott et al., 2006), decidimos iniciar un estudio para determinar la importancia de la sobreexpresión de *SETBP1* en LMA, así como del mecanismo molecular por el que ejerce su acción.

El gen *SETBP1* codifica una proteína de 1.542 aminoácidos y 170 kDa, de localización predominantemente nuclear, como hemos confirmado en este estudio. Tanto la función fisiológica como los mecanismos moleculares en los que SETBP1 participa son aún desconocidos. Contiene una región homóloga al dominio de dimerización de SKI, y una región de unión a SET, si bien el significado funcional de estas interacciones todavía no había sido determinado (Minakuchi et al., 2001). La proteína SET (I2PP2A/TAF-Iβ) inhibe a PP2A a través de la fosforilación de la tirosina 307 de su subunidad catalítica (Li et al., 1996). Además, SET se encuentra sobreexpresado en muchos tumores sólidos (Cervoni et al., 2002) así como en LMC (Neviani et al., 2005). En este trabajo, hemos demostrado que la sobreexpresión de *SETBP1* aumenta los niveles de la forma completa de 39 kDa de la proteína SET, probablemente debido a que la

interacción SETBP1-SET protege a SET del corte mediado por proteasas. Como consecuencia, detectamos una disminución de las formas SET de 27 kDa, 24 kDa y 20 kDa. Se ha descrito que SET inhibe la actividad DNasa del supresor tumoral NM23-H1, y que el corte de SET por granzyma A durante la apoptosis inducida por linfocitos T citotóxicos libera a NM23-H1 de esta inhibición, haciendo que transloque al núcleo donde corta el DNA (Beresford et al., 2001; Fan et al., 2003). El corte de SET por granzyma A genera tres polipéptidos de tamaños similares a los detectados en nuestro estudio, lo cual sugiere un posible papel de SETBP1 impidiendo el corte por granzyma A en la apoptosis independiente de caspasas inducida por los linfocitos T citotóxicos, en lo que sería un mecanismo de escape de la célula leucémica al ataque por linfocitos T citotóxicos.

PP2A es una proteína fosfatasa implicada en muchos procesos celulares (Janssens et al., 2001; Wang et al., 2004; Kawabe et al., 1997; Mumby, 2007, Westermarck et al., 2008), y su pérdida de función se ha asociado con transformación celular (Janssens et al., 2005; Schonthal, 2001). Teniendo en cuenta que SET es un potente inhibidor de PP2A, y que SETBP1 altera los niveles de SET, nos propusimos analizar los efectos de SETBP1 en PP2A. Además de los complejos previamente descritos SETBP1-SET y SET-PP2A, demostramos la formación de un complejo heterotrimérico SETBP1-SET-PP2A en el que PP2A se encuentra fosforilado y, por tanto, inhibido. Nuestros resultados sugieren que este es el mecanismo molecular por el cual PP2A está inhibido en pacientes con LMA y sobreexpresión de *SETBP1*, ya que PP2A y SETBP1 hemos comprobado que no interaccionan en ausencia de SET.

En el inmunoprecipitado del complejo SETBP1-SET-PP2A sólo se detectó la forma completa de SET, lo que indicaría que las formas procesadas cortas no participan en la formación de estos complejos. Se ha descrito que estas formas cortas conservan capacidad inhibitoria de PP2A, aunque no está claro si su actividad es igualmente potente (Beresford et al., 1999). En este sentido, nuestro trabajo indicaría que las formas procesadas presentan una menor actividad inhibitoria de PP2A, y que SETBP1 podría incrementar la inhibición de PP2A al aumentar la cantidad de proteína SET completa. Además, SET participa como modulador de procesos fundamentales como la replicación del DNA, remodelación de la cromatina y transcripción génica (Seo et al., 2001; Kutney et al., 2004), diferenciación (Kandilci et al., 2004), y control del ciclo celular (Canela et al., 2003). De este modo, el papel protector de la sobreexpresión de SETBP1 sobre SET podría dar lugar a cambios en los patrones de expresión de genes cuya acetilación dependa de la presencia de la forma completa de la proteína SET, ya que ésta y no las formas procesadas se ha visto que inhibe la acetilación de histonas y se une a HuR, el cual estabiliza los mRNAs de transcripción temprana (Cervoni et al., 2002; seo et al., 2001; Brennan et al., 2000). Por tanto, la interacción entre SETBP1 y SET podría tener otros efectos además de la inhibición de PP2A.

Para establecer la prevalencia y el significado pronóstico de la sobreexpresión de *SETBP1*, se analizaron sus niveles de expresión en una serie de 192 pacientes con LMA al diagnóstico, encontrando que la sobreexpresión de *SETBP1* es un evento recurrente en LMA que presenta una prevalencia del 27,6% de los casos estudiados. Los resultados obtenidos señalan que esta alteración predice una

menor supervivencia global (OS), con un impacto especialmente significativo en pacientes mayores de 60 años. La LMA es una enfermedad que se presenta a una edad media superior a los 60 años; sin embargo, los avances en el tratamiento de esta enfermedad se limitan al grupo de pacientes menores de 60 años, a pesar de que los mayores de 60 años representan al menos dos tercios del total de los casos de LMA (Buchner et al., 2009). Por lo tanto, es importante tanto la identificación de marcadores genéticos con valor pronóstico como el desarrollo de nuevas estrategias terapéuticas en este subgrupo de pacientes. En este sentido, nuestros datos señalan que la sobreexpresión de *SETBP1* podría distinguir dos subgrupos de pacientes mayores de 60 años con LMA y distinto pronóstico, y ser un factor predictivo de respuesta a activadores de PP2A, los cuales ya han sido propuestos como alternativa terapéutica para el tratamiento de LMC y LLA Philadelphia positiva (Neviani et al., 2007).

Aunque el análisis multivariante confirmó el impacto pronóstico negativo de *SETBP1* en nuestra serie de pacientes con LMA, esta alteración se asoció con otros marcadores de mal pronóstico como son la monosomía 7 y la sobreexpresión de *EVI1*. Por lo tanto, la activación de SETBP1 podría cooperar con otras alteraciones genéticas en la inducción de la LMA (Ott et al., 2006). En el paciente con la t(12;18) no se encontraron mutaciones en *FLT3*, *NPM1* ni *JAK2*, aunque la trisomía 19 observada en los blastos del mismo podría ser un evento secundario que estuviese cooperando en la progresión de la enfermedad. Por otro lado, las muestras en post-tratamiento tuvieron sobreexpresión de *SET* y *ETV6*, alteraciones que podrían estar cooperando con la sobreexpresión de *SETBP1*.

No sabemos los mecanismos moleculares mediante los cuales *SETBP1* se encuentra sobreexpresado en nuestra serie de pacientes. Sin embargo, el análisis bioinformático de la region promotora de *SETBP1* nos permitió identificar sitios de unión para varios factores de transcripción previamente implicados en leucemia, y cuya desregulación podría explicar la sobreexpresión de *SETBP1*. El estudio realizado por FISH sugiere que el punto de rotura en el paciente con la t(12;18) está localizado cerca del inicio de la transcripción de *SETBP1*, próximo a los sitios de inserción retroviral descritos por Ott et al. (2006). Además, 6 de las 7 inserciones retrovirales descritas en ese estudio están localizadas en una región de 200 pb, en donde hemos encontrado una isla CpG. Esto sugiere que se trata de una región importante para la regulación transcripcional del gen *SETBP1*, y que las alteraciones epigenéticas podrían ser otro mecanismo para la sobreexpresión de *SETBP1* en pacientes con LMA sin alteraciones cromosómicas en 18q.

En resumen, este trabajo describe un nuevo mecanismo de transformación leucémica. La sobreexpresión de SETBP1 lleva a un incremento de los niveles de proteína SET completa, inhibiendo la actividad fosfatasa del supresor tumoral PP2A mediante la formación de un complejo SETBP1-SET-PP2A, y promoviendo la proliferación celular. Además, hemos demostrado que la sobreexpresión de SETBP1 protege a SET del corte por proteasas, lo cual podría tener importantes efectos tanto en la actividad inhibitoria de SET sobre la acetilación de histonas, así como sobre la apoptosis independiente de caspasas mediada por granzyma A que inducen los linfocitos T citotóxicos. Por otra parte, la desregulación de *SETBP1* por translocaciones u otros mecanismos deconocidos parece jugar un papel importante en el desarrollo de la LMA. Hemos mostrado que la sobreexpresión de *SETBP1* es un evento molecular recurrente con valor

pronóstico independiente en LMA, especialmente en el subgrupo de pacientes con edad avanzada. Otros estudios dirigidos a estudiar la función fisiológica de este gen ayudarían a comprender mejor los múltiples pasos que llevan hasta el desarrollo de la LMA.

La desregulación de *SET* es un evento recurrente que contribuye a la inhibición de PP2A y que se asocia con peor pronóstico en leucemia mieloide aguda

La proteína SET es un potente inhibidor endógeno de PP2A que está sobreexpresado en distintos tumores, incluyendo leucemias BCR/ABL positivas (Neviani et al., 2005). Para comprobar si la desregulación de SET es un evento común en LMA, analizamos mediante QRT-PCR los niveles de expresión de *SET* en un panel de 13 líneas celulares BCR-ABL negativas de LMA, observando que la sobreexpresión de *SET* es un evento recurrente, aunque no siempre hay una buena correlación entre los niveles de mRNA y de proteína de SET.

Con objeto de evaluar la relevancia clínica de esta alteración, cuantificamos los niveles de expresión de *SET* por QRT-PCR en 214 pacientes con LMA al diagnóstico. Observamos que el 28% de los casos presentaban sobreexpresión de *SET*, y que se asociaba con el grupo citogenético de mal pronóstico ($p<0.01$), monosomía 7 ($p<0.01$), y con la sobreexpresión de *SETBP1* ($p<0.01$) y *EVI1* ($p=0.020$). Al estudiar el impacto pronóstico de la sobreexpresión de SET en 146 pacientes con LMA que habían recibido tratamiento de inducción, se observó que se trataba de un marcador independiente que se asocia significativamente con una menor supervivencia global (OS) y supervivencia libre de evento (EFS). Casi la mitad de los pacientes adultos con LMA eran citogenéticamente normales. Por lo tanto, la sobreexpresión de *SET* podría definir un subgrupo de pacientes con peor pronóstico dentro de este grupo con cariotipo normal. Aunque la mayoría de los pacientes con cariotipo normal pueden ser caracterizados individualmente en base a distintos marcadores moleculares, el tratamiento permanece todavía basado en una terapia citotóxica bastante inespecífica, lo que indica la necesidad de desarrollar nuevas terapias dirigidas a dianas concretas (Fathi et al., 2010; Zaidi et al., 2008). El análisis a nivel proteico en muestras de pacientes con LMA al diagnóstico confirmó la sobreexpresión de SET, aunque se detectaron algunos casos con sobreexpresión proteica que no la presentaban a nivel transcripcional. Esto indicaría que el análisis por QRT-PCR podría estar subestimando la recurrencia real de esta alteración en LMA.

En LMC se ha demostrado que BCR-ABL induce la sobreexpresión de SET y la consiguiente inhibición de PP2A (Eichhorn et al., 2009). La supresión de *SET* mediante shRNAs en líneas celulares BCR-ABL positivas provoca un incremento de la actividad PP2A y una reducción de su capacidad de inducir leucemia in vivo. Además, la sobreexpresión de SET confiere una ventaja proliferativa y es capaz de recuperar de forma completa la capacidad formadora de colonias independiente de citoquinas en células BCR-ABL positivas que expresan ectópicamente HA-PP2A (Neviani et al, 2005). Los resultados de nuestro estudio muestran que la sobreexpresón de SET en células de LMA induce proliferación celular, y también es capaz de revertir totalmente los efectos antiproliferativos

derivados de la expresión ectópica de PP2Ac. Sin embargo, sólo se consiguió una reversión parcial cuando las células fueron tratadas con forskolina, lo cual podría ser indicador de una toxicidad añadida de esta droga en células de LMA, independiente de la activación de PP2A.

Neviani et al. (2005) mostraron que la activación de BCR-ABL es el mecanismo que induce la sobreexpresión de SET en células BCR-ABL positivas. Nosotros nos propusimos investigar el mecanismo de sobreexpresión de SET en un modelo de LMA BCR-ABL negativo. En primer lugar estudiamos los niveles de expression del miR-199b en nuestras líneas celulares de LMA, ya que un trabajo reciente describía a *SET* como diana de este miRNA en coriocarcinoma (Chao et al., 2010). Sin embargo, EOL-1 fue la única línea celular en presentar niveles elevados de *SET* y bajos del miR-199b, por lo que la desregiulación del miR-199b se descartó como un mecanismo relevante responsable de los niveles elevados de *SET* en LMA. Las células transformadas presentan una gran variedad de mecanismos dirigidos a la inactivación de PP2A, entre los que se encuentran la presencia de alteraciones en subunidades estructurales, la sobreexpresión de inhibidores endógenos, y la supresión de subunidades reguladoras (Westermarck et al., 2008; Mumby, 2007; Eichhorn et al., 2009). Teniendo en cuenta que previamente nuestro grupo había descrito que varios de estos mecanismos pueden darse de forma simultánea en pacientes con LMA, nos planteamos la posibilidad de que el propio estado de activación de PP2A pudiera estar involucrado en la regulación de los niveles de *SET*. Al tratar células HEL con forskolina observamos una disminución de los niveles de *SET*, lo que indicaría que la activación de PP2A es capaz de reducir *SET*, sugiriendo que la pérdida de este control debido al estado de inhibición que PP2A presenta en LMA contribuiría a la desregulación de SET en esta enfermedad.

Por otra parte, el estudio bioinformático de la región promotor proximal de *SET* identificó varios sitios de unión para el factor de transcripción EVI1. La sobreexpresión de EVI1 es un factor de mal pronóstico en LMA y en nuestra serie, la sobreexpresión de *EVI1* y *SET* se asociaron significativamente, por lo que nos planteamos si el factor de transcripción EVI1 podría regular a *SET* a nivel transcripcional. El análisis mediante ChIP confirmó la unión de EVI1 al promotor de *SET*; sin embargo, los ensayos de luciferasa indicaron que *SET* no es una diana directa de EVI1, el cual necesitaría una maquinaria adicional para regular a *SET* o bien ejercería su regulación de una forma indirecta a través de otros mecanismos transcripcionales.

En resumen, en este trabajo hemos mostrado que la desregulación de SET es una alteración recurrente en LMA, que promueve la proliferación y revierte la reducción de la viabilidad celular inducida tras la expresión ectópica de PP2A. Además, el análisis de una serie de pacientes con LMA al diagnóstico indica que la sobreexpresión de *SET* es un marcador pronóstico independiente que se asocia con peor supervivencia global. Al investigar los mecanismos moleculares responsables de la sobreexpresión de *SET* se indentificó que el estado de activación de PP2A y el factor de transcripción EVI1 regulan de forma directa e indirecta, respectivamente, los niveles transcripcionales de *SET*. En definitiva, nuestra progresiva comprensión de los mecanismos moleculares en LMA podría conducir al desarrollo de nuevas alternativas terapéuticas que podrían mejorar la supervivencia de los pacientes con LMA. En este sentido, la sobreexpresión de

SET es capaz de discriminar un subgrupo de pacientes con peor pronóstico que podrían ser tratados con activadores de PP2A en futuros ensayos clínicos.

Valoración conjunta de los resultados obtenidos

El trabajo de investigación llevado a cabo durante el desarrollo de esta tesis doctoral ha permitido identificar nuevos mecanismos de desregulación génica en leucemia mieloide aguda.

En primer lugar, la integración de datos procedentes tanto de arrays de SNPs y expresión, como de los perfiles de miRNAs en 16 líneas mieloides ha permitido confirmar que la presencia de CNVs en regiones genómicas representa un mecanismo de desregulación de la expresión génica de miRNAs en LMA e identificar nuevos genes implicados en LMA. Así, este análisis experimental que ha incluido la integración de datos procedentes de arrays de SNPs, arrays de expresión y de los perfiles de expresión de miRNAs, constituye no sólo una forma de aclarar los mecanismos de desregulación de determinados genes en LMA, sino también nueva estrategia para descubrir nuevas dianas moleculares alteradas e importantes para el desarrollo de esta enfermedad.

En este sentido los resultados obtenidos han permitido identificar la sobreexpresión del miR-370 como un nuevo mecanismo de regulación del supresor tumoral *NF1*. Además, el miR-379 se identificó como un regulador negativo de los niveles de c-CBL, si bien en este caso la regulación sobre c-CBL se produce de forma indirecta a través de algún intermediario aún desconocido. La investigación del resto de genes candidatos encontrados en este estudio se encuentra actualmente e desarrollo.

Además, este trabajo ha permitido una mejor caracterización de las líneas celulares mieloides, las cuales han servido como herramienta indispensable para el desarrollo de nuestro objetivo principal de esta tesis doctoral: el estudio del papel de PP2A en la leucemia mieloide aguda.

Nuestro proyecto de investigación acerca del papel de PP2A en LMA surgió en primera instancia como consecuencia del análisis de una nueva traslocación t(12;18) identificada en un paciente con LMA-M5 secundaria a síndrome mielodisplásico. El estudio de esta nueva traslocación reveló que el evento importante en la misma era la sobreexpresión del gen *SETBP1* debido a que el punto de rotura en el cromosoma 18 se encontraba muy próximo al comienzo de este gen, afectando a la regulación transcripcional del mismo. A pesar de tratarse de un gen cuya función fisiológica era desconocida, un trabajo reciente había descrito que su sobreexpresión debido a inserciones retrovirales localizadas en la región próxima a su inicio, otorgaba una ventaja proliferativa a las células (Ott et al., 2006). El estudio del mecanismo molecular por el que SETBP1 ejerce su acción nos permitió identificar que SETBP1 se une a SET formando un heterodímero que protege a SET del corte por proteasas. Esto lleva a un incremento de proteína SET completa y a la formación de complejos heterotriméricos SETBP1-SET-PP2A en los que PP2A queda inhibido, promoviéndose de este modo la proliferación celular. Además, comprobamos que

la sobreexpresión de *SETBP1* es un evento recurrente en LMA, y un marcador independiente de mal pronóstico en pacientes mayores de 60 años.

Debido a su recurrencia (27%) y a su efecto sobre la actividad de PP2A, nos planteamos que la inhibición de PP2A podría ser una alteración común en LMA. Por otra parte, la inactivación de PP2A había sido descrita en diversos tipos de cáncer (Mumby, 2007; Sablina et al., 2007; Junttila et al., 2008). En leucemias, se había descrito que su inactivación era esencial tanto en LMC como en LLA BCR-ABL+, y que su activación tenía un potencial valor terapéutico en estas enfermedades. En estos casos, el mecanismo de inactivación de PP2A es a través de BCR-ABL, el cual induce un aumento de los niveles de SET, un potente inhibidor endógeno de PP2A (Neviani et al., 2005; Neviani et al., 2007). Además, también se había descrito la inactivación de PP2A en LLC-B y en leucemias c-KIT+ (Liu et al., 2008; Roberts et al., 2010). Nuestros resultados señalan que la inactivación de PP2A es recurrente en LMA y que su activación farmacológica mediante forskolina ó FTY70 bloquea la proliferación celular, induce apoptosis dependiente de caspasas, y afecta al estado de activación de AKT y ERK. Resulta interesante que el tratamiento con estas drogas activadoras de PP2A presentó un efecto aditivo con Idarrubicina y Ara-c, drogas empleadas en el tratamiento éstardar de inducción en pacientes con LMA, lo que indicaría el potencial valor terapéutico de un futuro tratamiento combinado con ambos tipos de drogas en LMA.

Teniendo en cuenta que la LMA es un modelo BCR-ABL negativo, nos planteamos estudiar los mecanismos de inhibición de PP2A en esta enfermedad. Así, identificamos la sobreexpresión de inhibidores endógenos de PP2A como *CIP2A* (Junttila et al., 2007) y *SET* (Li et al., 1996), así como la expresión reducida de ciertas subunidades de PP2A, como la catalítica, las subunidades reguladoras *PPP2R5B* y *PPP2R5C*, y la subunidad estructural *PPP2R1B*. Curiosamente, se encontraron varios de estos mecanismos actuando simultáneamente, indicando que son mecanismos que pueden cooperar entre sí en el mismo paciente. También se identificaron algunos casos que presentaron inhibición de PP2A pero ninguno de los mecanismos descritos, lo que sugiere la existencia de algún otro mecanismo de inactivación de PP2A que todavía no ha sido identificado.

Debido a que SET ejerce funciones muy importantes dentro de la célula y que se ha descrito sobreexpresado en distintos tipos de tumores (revisado en Westermarck et al., 2008), nos planteamos comprobar la importancia de su sobreexpresión en LMA. Así, la cuantificación de la expresión de *SET* en 214 muestras de pacientes con LMA al diagnóstico reveló que esta alteración presenta una prevalencia del 28% y que se asocia con peor pronóstico, especialmente dentro del grupo de pacientes con cariotipo normal. Además, el análisis a nivel proteico en 16 pacientes de esta serie mostró que la prevalencia de la sobreexpresión de *SET* podría ser mayor de lo que indica el análisis a nivel transcripcional, ya que en algunos pacientes en los que no se detectó la alteración a nivel de mRNA si presentaron niveles aumentados de proteína SET. Por otra parte, la expresión ectópica de SET en células AML es capaz de inducir un aumento de la proliferación, y de revertir el bloqueo de la misma tras la expresión ectópica de PP2Ac. Entre los mecanismos responsables de la sobreexpresión de

SET podría estar la propia inhibición de PP2A, ya que su activación farmacológica con forskolina induce una reducción en los niveles de *SET*.

En resumen, este proyecto ha permitido determinar que la inhibición de PP2A es un mecanismo recurrente en LMA y la identificación de los mecanismos moleculares responsables de dicha inactivación. Además, se ha comprobado que su activación farmacológica, al igual que lo desccrito para otros tipos de leucemias, tiene un potencial valor terapéutico en leucemia mieloide aguda (Figura 1).

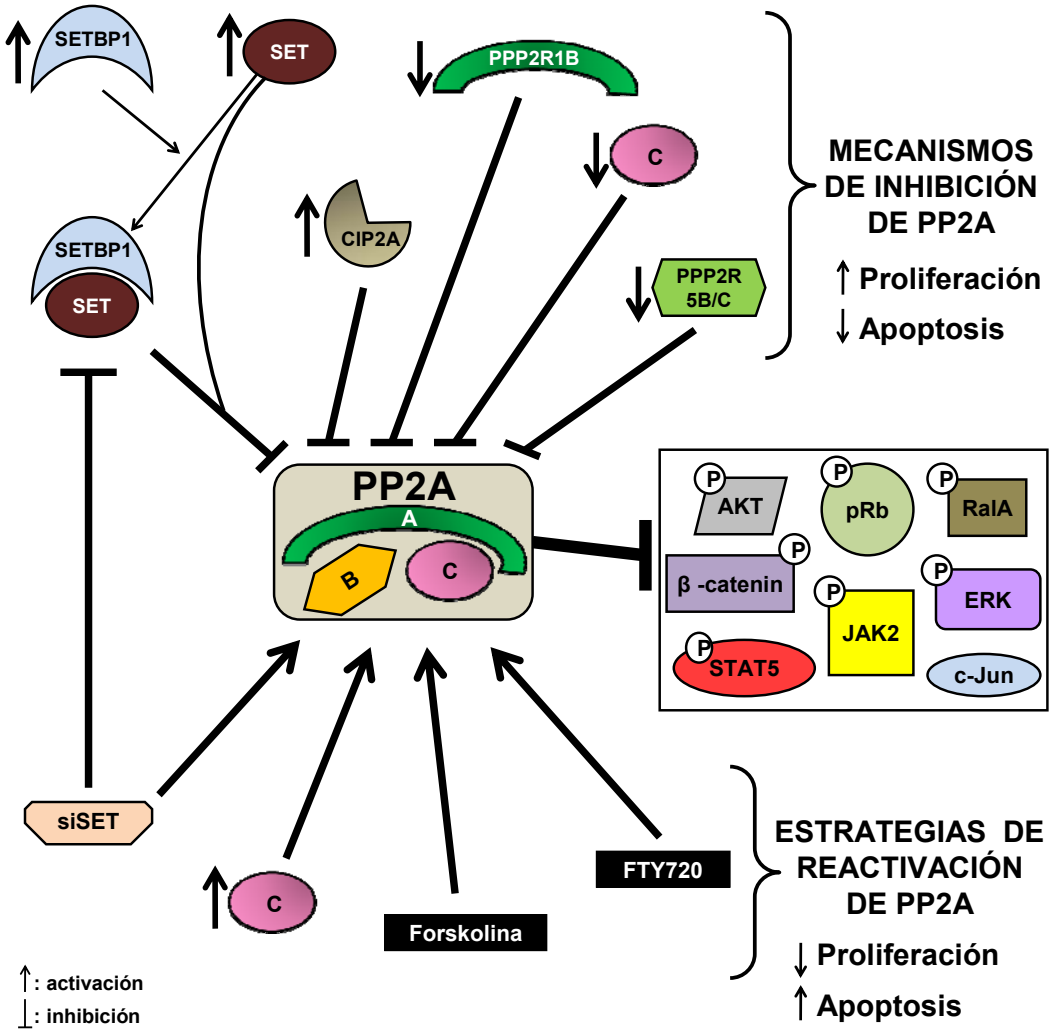


Figura 1. Modelo que incluye los distintos mecanismos de inhibición de PP2A identificados en células de leucemia mieloide aguda, así como las diferentes estrategias de activación de PP2A.

CONCLUSIONES

1. Se han identificado 19 miRNAs cuya expresión se correlaciona significativamente con la presencia de variaciones de número de copia en la región cromosómica en la que se sitúan: 16 de ellos sobreexpresados y localizados en regiones con amplificación, y 3 de ellos con expresión reducida y localizados en regiones con delección. Por tanto, la desregulación de miRNAs debido a su localización en regiones afectadas por alteraciones genómicas representa un mecanismo alternativo de regulación de los niveles de expresión génica en leucemia mieloide aguda.

2. Se ha identificado al gen *NF1* como diana directa del miR-370. La expresión de *NF1* se mantiene reducida debido a que el miR-370, localizado en una región de amplificación, se encuentra sobreexpresado.

3. La inactivación de PP2A es un evento recurrente en pacientes con leucemia mieloide aguda y su activación farmacológica en líneas celulares bloquea la proliferación e induce apoptosis. Además, muestra un efecto aditivo con Idarrubicina y Ara-c, fármacos empleados en el tratamiento de inducción de pacientes con leucemia mieloide aguda. Por tanto, el empleo de activadores de PP2A podría representar una alternativa terapéutica en leucemia mieloide aguda, solo o en combinación con drogas usadas en terapia de inducción.

4. La inactivación de PP2A en pacientes con leucemia mieloide aguda se lleva a cabo a través de distintos mecanismos: la disminución de la expresión proteica de subunidades de PP2A, como la catalítica, la subunidad estructural *PPP2R1B* y las subunidades reguladoras *PPP2R5B* y *PPP2R5C*, y la sobreexpresión de inhibidores endógenos de PP2A, como *SET* y *CIP2A*.

5. La sobreexpresión de *SETBP1* es una alteración recurrente en leucemia mieloide aguda, que se asocia con el grupo de mal pronóstico citogenético, y es un marcador independiente de mal pronóstico en pacientes mayores de 60 años.

6. SETBP1 ejerce su acción a través de un mecanismo molecular que consiste en proteger a SET del corte por proteasas, aumentando de este modo la cantidad de proteína SET completa, lo que lleva a la formación de complejos heterotriméricos SETBP1-SET-PP2A a través de los cuales SET inhibe la actividad de PP2A, aumentando la proliferación celular.

7. La desregulación de SET es un evento recurrente en leucemia mieloide aguda tanto a nivel transcripcional como proteico, y su sobreexpresión es un marcador independiente que se asocia con peor pronóstico en esta enfermedad.

8. La sobreexpresión de SET induce un aumento de proliferación y es capaz de revertir el efecto antiproliferativo tras la expresión ectópica de PP2A. Además, la activación de PP2A reduce los niveles de expresión de SET, lo que indica que el estado de inhibición de PP2A en células leucémicas sería un mecanismo que contribuye a la sobreexpresión de SET.

BIBLIOGRAFÍA

Adams DG, Coffee RL, Jr., Zhang H, Pelech S, Strack S, Wadzinski BE. Positive regulation of Raf1-MEK1/2-ERK1/2 signaling by protein serine/threonine phosphatase 2A holoenzymes. *J Biol Chem*. 2005;280(52):42644-42654.

Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol*. 2005;6(8):635-645.

Adler HT, Nallaseth FS, Walter G, Tkachuk DC. HRX leukemic fusion proteins form a heterocomplex with the leukemia-associated protein SET and protein phosphatase 2A. *J Biol Chem*. 1997;272(45):28407-28414.

Al-Murrani SW, Woodgett JR, Damuni Z. Expression of I2PP2A, an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity. *Biochem J*. 1999;341 (Pt 2):293-298.

Ambs S, Prueitt RL, Yi M, et al. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Res*. 2008;68(15):6162-6170.

Andersson A, Ritz C, Lindgren D, et al. Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal residual disease status. *Leukemia*. 2007;21(6):1198-1203.

Andrabi S, Gjoerup OV, Kean JA, Roberts TM, Schaffhausen B. Protein phosphatase 2A regulates life and death decisions via Akt in a context-dependent manner. *Proc Natl Acad Sci U S A*. 2007;104(48):19011-19016.

Arino J, Woon CW, Brautigan DL, Miller TB, Jr., Johnson GL. Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. *Proc Natl Acad Sci U S A*. 1988;85(12):4252-4256.

Arnold HK, Sears RC. Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. *Mol Cell Biol*. 2006;26(7):2832-2844.

Arroyo JD, Hahn WC. Involvement of PP2A in viral and cellular transformation. *Oncogene*. 2005;24(52):7746-7755.

Azuma H, Takahara S, Ichimaru N, et al. Marked prevention of tumor growth and metastasis by a novel immunosuppressive agent, FTY720, in mouse breast cancer models. *Cancer Res*. 2002;62(5):1410-1419.

Babashah S, Jamali S, Mahdian R, et al. Detection of unknown deletions in beta-globin gene cluster using relative quantitative PCR methods. *Eur J Haematol*. 2009;83(3):261-269.

Baharians Z, Schonthal AH. Autoregulation of protein phosphatase type 2A expression. *J Biol Chem*. 1998;273(30):19019-19024.

Bandres E, Cubedo E, Agirre X, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Mol cancer*. 2006;5:29.

Barker N, Clevers H. Catenins, Wnt signaling and cancer. *Bioessays*. 2000;22(11):961-965.

Becker H, Marcucci G, Maharry K, et al. Favorable prognostic impact of NPM1 mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2010;28(4):596-604.

Bellacosa A, de Feo D, Godwin AK, et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int J Cancer*. 1995;64(4):280-285.

Beresford PJ, Xia Z, Greenberg AH, Lieberman J. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity*. 1999;10(5):585-594.

Beresford PJ, Zhang D, Oh DY, et al. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem*. 2001;276(46):43285-43293.

Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res*. 2008;36(Database issue):D149-153.

Bialojan C, Takai A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem J*. 1988;256(1):283-290.

Bosch M, Cayla X, Van Hoof C, et al. The PR55 and PR65 subunits of protein phosphatase 2A from Xenopus laevis. molecular cloning and developmental regulation of expression. *Eur J Biochem*. 1995;230(3):1037-1045.

Brennan CM, Gallouzi IE, Steitz JA. Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J Cell Biol*. 2000;151(1):1-14.

Bryant JC, Westphal RS, Wadzinski BE. Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit. *Biochem J*. 1999;339 (Pt 2):241-246.

Bryne JC, Valen E, Tang MH, et al. JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic Acids Res*. 2008;36(Database issue):D102-106.

Buchner T, Berdel WE, Haferlach C, et al. Age-related risk profile and chemotherapy dose response in acute myeloid leukemia: a study by the German Acute Myeloid Leukemia Cooperative Group. *J Clin Oncol*. 2009;27(1):61-69.

Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1605-1616.

Burnett A, Wetzler M, Löwenberg B. Therapeutic advances in acute myeloid leukemia. *J Clin Oncol*. 2011;29(5):487-94.

Calabretta B, Perrotti D. The biology of CML blast crisis. *Blood*. 2004;103(11):4010-4022.

Calin GA, di Iasio MG, Caprini E, et al. Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms. *Oncogene*. 2000;19(9):1191-1195.

Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*. 2004;101(9):2999-3004.

Canela N, Rodriguez-Vilarrupla A, Estanyol JM, et al. The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. *J Biol Chem*. 2003;278(2):1158-1164.

Carlson SG, Eng E, Kim EG, Perlman EJ, Copeland TD, Ballermann BJ. Expression of SET, an inhibitor of protein phosphatase 2A, in renal development and Wilms' tumor. *J Am Soc Nephrol*. 1998;9(10):1873-1880.

Cayla X, Goris J, Hermann J, Hendrix P, Ozon R, Merlevede W. Isolation and characterization of a tyrosyl phosphatase activator from rabbit skeletal muscle and Xenopus laevis oocytes. *Biochemistry*. 1990;29(3):658-667.

Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. *J Biol Chem*. 2002;277(28):25026-25031.

Chao A, Tsai CL, Wei PC, et al. Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. *Cancer Lett*. 2010;291(1):99-107.

Chao RC, Pyzel U, Fridlyand J, et al. Therapy-induced malignant neoplasms in Nf1 mutant mice. *Cancer Cell*. 2005;8(4):337-348.

Chao TS, Abe M, Hershenson MB, Gomes I, Rosner MR. Src tyrosine kinase mediates stimulation of Raf-1 and mitogen-activated protein kinase by the tumor promoter thapsigargin. *Cancer Res*. 1997;57(15):3168-3173.

Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science*. 1992;257(5074):1261-1264.

Chen KF, Liu CY, Lin YC, et al. CIP2A mediates effects of bortezomib on phospho-Akt and apoptosis in hepatocellular carcinoma cells. *Oncogene*. 2010;29(47):6257-6266.

Chen W, Arroyo JD, Timmons JC, Possemato R, Hahn WC. Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. *Cancer Res*. 2005;65(18):8183-8192.

Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell*. 2004;5(2):127-136.

Chiang CW, Kanies C, Kim KW, et al. Protein phosphatase 2A dephosphorylation of phosphoserine 112 plays the gatekeeper role for BAD-mediated apoptosis. *Mol Cell Biol*. 2003;23(18):6350-6362.

Chien Y, White MA. RAL GTPases are linchpin modulators of human tumour-cell proliferation and survival. *EMBO Rep*. 2003;4(8):800-806.

Cho US, Xu W. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. *Nature*. 2007;445(7123):53-57.

Chou HC, Chen CH, Lee HS, et al. Alterations of tumour suppressor gene PPP2R1B in hepatocellular carcinoma. *Cancer Lett*. 2007;253(1):138-143.

Coenen EA, Zwaan CM, Meyer C, et al. KIAA1524: A novel MLL translocation partner in acute myeloid leukemia. *Leuk Res*. 2011;35(1):133-135.

Coessens B, Thijs G, Aerts S, et al. INCLUSive: A web portal and service registry for microarray and regulatory sequence analysis. *Nucleic Acids Res*. 2003;31(13):3468-3470.

Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem*. 1989;58:453-508.

Come C, Laine A, Chanrion M, et al. CIP2A is associated with human breast cancer aggressivity. *Clin Cancer Res*. 2009;15(16):5092-5100.

Cools J, Mentens N, Odero MD, et al. Evidence for position effects as a variant ETV6-mediated leukemogenic mechanism in myeloid leukemias with a t(4;12)(q11-q12;p13) or t(5;12)(q31;p13). *Blood*. 2002;99(5):1776-1784.

Creyghton MP, Roel G, Eichhorn PJ, et al. PR72, a novel regulator of Wnt signaling required for Naked cuticle function. *Genes Dev*. 2005;19(3):376-386.

Creyghton MP, Roel G, Eichhorn PJ, Vredeveld LC, Destree O, Bernards R. PR130 is a modulator of the Wnt-signaling cascade that counters repression of the antagonist Naked cuticle. *Proc Natl Acad Sci U S A*. 2006;103(14):5397-5402.

Cristobal I, Blanco FJ, Garcia-Orti L, et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood*. 2010;115(3):615-625.

Cristobal I, Garcia-Orti L, Cirauqui C, Alonso MM, Calasanz MJ, Odero MD. PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect. *Leukemia*. 2011. [Epub ahead of print].

Csortos C, Zolnierowicz S, Bako E, Durbin SD, DePaoli-Roach AA. High complexity in the expression of the B' subunit of protein phosphatase 2A0. Evidence for the existence of at least seven novel isoforms. *J Biol Chem*. 1996;271(5):2578-2588.

Cutts BA, Sjogren AK, Andersson KM, et al. Nf1 deficiency cooperates with oncogenic K-RAS to induce acute myeloid leukemia in mice. *Blood*. 2009;114(17):3629-3632.

Dancey J, Sausville EA. Issues and progress with protein kinase inhibitors for cancer treatment. *Nat Rev Drug Discov*. 2003;2(4):296-313.

Deichmann M, Polychronidis M, Wacker J, Thome M, Naher H. The protein phosphatase 2A subunit Bgamma gene is identified to be differentially expressed in malignant melanomas by subtractive suppression hybridization. *Melanoma Res*. 2001;11(6):577-585.

Deschler B, Lubbert M. Acute myeloid leukemia: epidemiology and etiology. *Cancer*. 2006;107(9):2099-2107.

Dixon-McIver A, East P, Mein CA, et al. Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia. *PLoS One*. 2008;3(5):e2141.

Dohner H. Implication of the molecular characterization of acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*. 2007:412-419.

Döhner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*. 2010;115(3):453-74.

Dong QZ, Wang Y, Dong XJ, et al. CIP2A is Overexpressed in Non-Small Cell Lung Cancer and Correlates with Poor Prognosis. *Ann Surg Oncol*. 2011;18(3):857-865.

Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta*. 2009;1795(1):1-15.

Eichhorn PJ, Creyghton MP, Wilhelmsen K, van Dam H, Bernards R. A RNA interference screen identifies the protein phosphatase 2A subunit PR55gamma as a stress-sensitive inhibitor of c-SRC. *PLoS Genet*. 2007;3(12):e218.

Esplin ED, Ramos P, Martinez B, Tomlinson GE, Mumby MC, Evans GA. The glycine 90 to aspartate alteration in the Abeta subunit of PP2A (PPP2R1B) associates with breast cancer and causes a deficit in protein function. *Genes Chromosomes Cancer*. 2006;45(2):182-190.

Estey E, Dohner H. Acute myeloid leukaemia. *Lancet*. 2006;368(9550):1894-1907.

Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell*. 2003;112(5):659-672.

Fathi AT, Grant S, Karp JE. Exploiting cellular pathways to develop new treatment strategies for AML. *Cancer Treat Rev*. 2010;36(2):142-150.

Fellner T, Lackner DH, Hombauer H, et al. A novel and essential mechanism determining specificity and activity of protein phosphatase 2A (PP2A) in vivo. *Genes Dev*. 2003;17(17):2138-2150.

Ferrajoli A, Faderl S, Van Q, et al. WP1066 disrupts Janus kinase-2 and induces caspase-dependent apoptosis in acute myelogenous leukemia cells. *Cancer Res*. 2007;67(23):11291-11299.

Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19(1):92-105.

Fujiki H, Suganuma M. Tumor promotion by inhibitors of protein phosphatases 1 and 2A: the okadaic acid class of compounds. *Adv Cancer Res*. 1993;61:143-194.

Gallay N, Dos Santos C, Cuzin L, et al. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia*. 2009;23(6):1029-1038.

Garzon R, Croce CM. MicroRNAs in normal and malignant hematopoiesis. *Curr Opin Hematol*. 2008;15(4):352-358.

Garzon R, Garofalo M, Martelli MP, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A*. 2008;105(10):3945-3950.

Gildea JJ, Harding MA, Seraj MJ, Gulding KM, Theodorescu D. The role of Ral A in epidermal growth factor receptor-regulated cell motility. *Cancer Res*. 2002;62(4):982-985.

Gilliland DG, Griffin JD. Role of FLT3 in leukemia. *Curr Opin Hematol*. 2002;9(4):274-281.

Ginos MA, Page GP, Michalowicz BS, et al. Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. *Cancer Res*. 2004;64(1):55-63.

Goi T, Shipitsin M, Lu Z, Foster DA, Klinz SG, Feig LA. An EGF receptor/Ral-GTPase signaling cascade regulates c-Src activity and substrate specificity. *EMBO J*. 2000;19(4):623-630.

Goris J, Pallen CJ, Parker PJ, Hermann J, Waterfield MD, Merlevede W. Conversion of a phosphoseryl/threonyl phosphatase into a phosphotyrosyl phosphatase. *Biochem J*. 1988;256(3):1029-1034.

Gotz J, Probst A, Ehler E, Hemmings B, Kues W. Delayed embryonic lethality in mice lacking protein phosphatase 2A catalytic subunit Calpha. *Proc Natl Acad Sci U S A*. 1998;95(21):12370-12375.

Grady WM, Parkin RK, Mitchell PS, et al. Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. *Oncogene*. 2008;27(27):3880-3888.

Gregory MA, Hann SR. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol Cell Biol*. 2000;20(7):2423-2435.

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res*. 2008;36(Database issue):D154-158.

Grimwade D, Hills RK. Independent prognostic factors for AML outcome. *Hematology Am Soc Hematol Educ Program*. 2009:385-395.

Groves MR, Hanlon N, Turowski P, Hemmings BA, Barford D. The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. *Cell*. 1999;96(1):99-110.

Haferlach T. Molecular genetic pathways as therapeutic targets in acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*. 2008:400-411.

Hahn WC, Weinberg RA. Rules for making human tumor cells. *N Engl J Med*. 2002;347(20):1593-1603.

Hamad NM, Elconin JH, Karnoub AE, et al. Distinct requirements for Ras oncogenesis in human versus mouse cells. *Genes Dev*. 2002;16(16):2045-2057.

Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.

Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature*. 1997;387(6630):296-299.

Hemmer S, Wasenius VM, Haglund C, et al. Alterations in the suppressor gene PPP2R1B in parathyroid hyperplasias and adenomas. *Cancer Genet Cytogenet*. 2002;134(1):13-17.

Hemmings BA, Adams-Pearson C, Maurer F, et al. alpha- and beta-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry*. 1990;29(13):3166-3173.

Hendrix P, Mayer-Jackel RE, Cron P, et al. Structure and expression of a 72-kDa regulatory subunit of protein phosphatase 2A. Evidence for different size forms produced by alternative splicing. *J Biol Chem*. 1993;268(20):15267-15276.

Hendrix P, Turowski P, Mayer-Jaekel RE, et al. Analysis of subunit isoforms in protein phosphatase 2A holoenzymes from rabbit and Xenopus. *J Biol Chem*. 1993;268(10):7330-7337.

Hombauer H, Weismann D, Mudrak I, et al. Generation of active protein phosphatase 2A is coupled to holoenzyme assembly. *PLoS Biol*. 2007;5(6):e155.

Hunter T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell*. 1995;80(2):225-236.

Ikura M. Calcium binding and conformational response in EF-hand proteins. *Trends Biochem Sci*. 1996;21(1):14-17.

Iliopoulos D, Drosatos K, Hiyama Y, Goldberg IJ, Zannis VI. MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism. *J Lipid Res*. 2010;51(6):1513-1526.

Ingram DA, Yang FC, Travers JB, et al. Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumor suppressor gene modulates melanocyte and mast cell fates in vivo. *J Exp Med*. 2000;191(1):181-188.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*. 2003;31(4):e15.

Ito A, Kataoka TR, Watanabe M, et al. A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. *EMBO J*. 2000;19(4):562-571.

Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. *Curr Opin Genet Dev*. 2005;15(1):34-41.

Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J*. 2001;353(Pt 3):417-439.

Janssens V, Jordens J, Stevens I, et al. Identification and functional analysis of two Ca2+-binding EF-hand motifs in the B"/PR72 subunit of protein phosphatase 2A. *J Biol Chem*. 2003;278(12):10697-10706.

Janssens V, van Hoof C, Martens E, de Baere I, Merlevede W, Goris J. Identification and characterization of alternative splice products encoded by the human phosphotyrosyl phosphatase activator gene. *Eur J Biochem*. 2000;267(14):4406-4413.

Jin Z, Wallace L, Harper SQ, Yang J. PP2A:B56{epsilon}, a substrate of caspase-3, regulates p53-dependent and p53-independent apoptosis during development. *J Biol Chem*. 2010;285(45):34493-34502.

Junttila MR, Li SP, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB J*. 2008;22(4):954-965.

Junttila MR, Puustinen P, Niemela M, et al. CIP2A inhibits PP2A in human malignancies. *Cell*. 2007;130(1):51-62.

Junttila MR, Westermarck J. Mechanisms of MYC stabilization in human malignancies. *Cell Cycle*. 2008;7(5):592-596.

Kales SC, Ryan PE, Nau MM, Lipkowitz S. Cbl and human myeloid neoplasms: the Cbl oncogene comes of age. *Cancer Res*. 2010;70(12):4789-4794.

Kalla C, Scheuermann MO, Kube I, et al. Analysis of 11q22-q23 deletion target genes in B-cell chronic lymphocytic leukaemia: evidence for a pathogenic role of NPAT, CUL5, and PPP2R1B. *Eur J Cancer*. 2007;43(8):1328-1335.

Kandilci A, Mientjes E, Grosveld G. Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937. *Leukemia*. 2004;18(2):337-340.

Katayose Y, Li M, Al-Murrani SW, Shenolikar S, Damuni Z. Protein phosphatase 2A inhibitors, I(1)(PP2A) and I(2)(PP2A), associate with and modify the substrate specificity of protein phosphatase 1. *J Biol Chem*. 2000;275(13):9209-9214.

Katoh F, Fitzgerald DJ, Giroldi L, Fujiki H, Sugimura T, Yamasaki H. Okadaic acid and phorbol esters: comparative effects of these tumor promoters on cell transformation, intercellular communication and differentiation in vitro. *Jpn J Cancer Res*. 1990;81(6-7):590-597.

Kawabe T, Muslin AJ, Korsmeyer SJ. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. *Nature*. 1997;385(6615):454-458.

Kerosuo L, Fox H, Perala N, et al. CIP2A increases self-renewal and is linked to Myc in neural progenitor cells. *Differentiation*. 2010;80(1):68-77.

Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in microRNA target recognition. *Nat Genet*. 2007;39(10):1278-1284.

Khanna A, Bockelman C, Hemmes A, et al. MYC-dependent regulation and prognostic role of CIP2A in gastric cancer. *J Natl Cancer Inst*. 2009;101(11):793-805.

Khew-Goodall Y, Hemmings BA. Tissue-specific expression of mRNAs encoding alpha- and beta-catalytic subunits of protein phosphatase 2A. *FEBS Lett*. 1988;238(2):265-268.

Kong M, Fox CJ, Mu J, et al. The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis. *Science*. 2004;306(5696):695-698.

Korkola JE, Houldsworth J, Chadalavada RS, et al. Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with in vivo differentiation of human male germ cell tumors. *Cancer Res*. 2006;66(2):820-827.

Kornblau SM, Singh N, Qiu Y, Chen W, Zhang N, Coombes KR. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. *Clin Cancer Res*. 2010;16(6):1865-1874.

Kuo YC, Huang KY, Yang CH, Yang YS, Lee WY, Chiang CW. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. *J Biol Chem*. 2008;283(4):1882-1892.

Kutney SN, Hong R, Macfarlan T, Chakravarti D. A signaling role of histone-binding proteins and INHAT subunits pp32 and Set/TAF-Ibeta in integrating chromatin hypoacetylation and transcriptional repression. *J Biol Chem*. 2004;279(29):30850-30855.

Lall S, Grun D, Krek A, et al. A genome-wide map of conserved microRNA targets in C. elegans. *Curr Biol*. 2006;16(5):460-471.

Lee TK, Man K, Ho JW, et al. FTY720: a promising agent for treatment of metastatic hepatocellular carcinoma. *Clin Cancer Res*. 2005;11(23):8458-8466.

Lee TY, Lai TY, Lin SC, et al. The B56gamma3 regulatory subunit of protein phosphatase 2A (PP2A) regulates S phase-specific nuclear accumulation of PP2A and the G1 to S transition. *J Biol Chem*. 2010;285(28):21567-21580.

Letourneux C, Rocher G, Porteu F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *EMBO J*. 2006;25(4):727-738.

Levis MJ. Will newer tyrosine kinase inhibitors have an impact in AML? *Best Pract Res Clin Haematol*. 2010;23(4):489-494.

Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*. 2008;456(7218):66-72.

Li HH, Cai X, Shouse GP, Piluso LG, Liu X. A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J*. 2007;26(2):402-411.

Li M, Guo H, Damuni Z. Purification and characterization of two potent heat-stable protein inhibitors of protein phosphatase 2A from bovine kidney. *Biochemistry*. 1995;34(6):1988-1996.

Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem*. 1996;271(19):11059-11062.

Li W, Ge Z, Liu C, et al. CIP2A is overexpressed in gastric cancer and its depletion leads to impaired clonogenicity, senescence, or differentiation of tumor cells. *Clin Cancer Res*. 2008;14(12):3722-3728.

Li X, Virshup DM. Two conserved domains in regulatory B subunits mediate binding to the A subunit of protein phosphatase 2A. *Eur J Biochem*. 2002;269(2):546-552.

Li Z, Lu J, Sun M, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. *Proc Natl Acad Sci U S A*. 2008;105(40):15535-15540.

Lim KH, Baines AT, Fiordalisi JJ, et al. Activation of RalA is critical for Ras-induced tumorigenesis of human cells. *Cancer Cell*. 2005;7(6):533-545.

Liu Q, Zhao X, Frissora F, et al. FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. *Blood*. 2008;111(1):275-284.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.

Loh ML. Childhood myelodysplastic syndrome: focus on the approach to diagnosis and treatment of juvenile myelomonocytic leukemia. *Hematology Am Soc Hematol Educ Program*. 2010;2010:357-362.

Longin S, Jordens J, Martens E, et al. An inactive protein phosphatase 2A population is associated with methylesterase and can be re-activated by the phosphotyrosyl phosphatase activator. *Biochem J*. 2004;380(Pt 1):111-119.

Longin S, Zwaenepoel K, Louis JV, Dilworth S, Goris J, Janssens V. Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit. *J Biol Chem*. 2007;282(37):26971-26980.

Loriaux MM, Levine RL, Tyner JW, et al. High-throughput sequence analysis of the tyrosine kinome in acute myeloid leukemia. *Blood*. 2008;111(9):4788-4796.

Luo LY, Huang J, Gou BD, Zhang TL, Wang K. Induction of human promyelocytic leukemia HL-60 cell differentiation into monocytes by arsenic sulphide: involvement of serine/threonine protein phosphatases. *Leuk Res*. 2006;30(11):1399-1405.

Ma J, Arnold HK, Lilly MB, Sears RC, Kraft AS. Negative regulation of Pim-1 protein kinase levels by the B56beta subunit of PP2A. *Oncogene*. 2007;26(35):5145-5153.

Malempati S, Tibbitts D, Cunningham M, et al. Aberrant stabilization of c-Myc protein in some lymphoblastic leukemias. *Leukemia*. 2006;20(9):1572-1581.

Marcucci G, Haferlach T, Döhner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. *J Clin Oncol*. 2011;29(5):475-86.

Marcucci G, Mrozek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. *Blood*. 2011;117(4):1121-1129.

Marcucci G, Radmacher MD, Mrozek K, Bloomfield CD. MicroRNA expression in acute myeloid leukemia. *Curr Hematol Malig Rep*. 2009;4(2):83-88.

Margolis SS, Perry JA, Forester CM, et al. Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell*. 2006;127(4):759-773.

Martelli AM, Papa V, Tazzari PL, et al. Erucylphosphohomocholine, the first intravenously applicable alkylphosphocholine, is cytotoxic to acute myelogenous leukemia cells through JNK- and PP2A-dependent mechanisms. *Leukemia*. 2010;24(4):687-698.

Matys V, Kel-Margoulis OV, Fricke E, et al. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res*. 2006;34(Database issue):D108-110.

Mavrakis KJ, Wolfe AL, Oricchio E, et al. Genome-wide RNA-mediated interference screen identifies miR-19 targets in Notch-induced T-cell acute lymphoblastic leukaemia. *Nat Cell Biol*. 2010;12(4):372-379.

Mayer RE, Hendrix P, Cron P, et al. Structure of the 55-kDa regulatory subunit of protein phosphatase 2A: evidence for a neuronal-specific isoform. *Biochemistry*. 1991;30(15):3589-3597.

McConnell JL, Gomez RJ, McCorvey LR, Law BK, Wadzinski BE. Identification of a PP2A-interacting protein that functions as a negative regulator of phosphatase activity in the ATM/ATR signaling pathway. *Oncogene*. 2007;26(41):6021-6030.

McCright B, Virshup DM. Identification of a new family of protein phosphatase 2A regulatory subunits. *J Biol Chem*. 1995;270(44):26123-26128.

McCright IJ, Tsunoda I, Whitby FG, Fujinami RS. Theiler's viruses with mutations in loop I of VP1 lead to altered tropism and pathogenesis. *J Virol*. 1999;73(4):2814-2824.

Meng F, Wehbe-Janek H, Henson R, Smith H, Patel T. Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes. *Oncogene*. 2008;27(3):378-386.

Millward TA, Zolnierowicz S, Hemmings BA. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem Sci*. 1999;24(5):186-191.

Minakuchi M, Kakazu N, Gorrin-Rivas MJ, et al. Identification and characterization of SEB, a novel protein that binds to the acute undifferentiated leukemia-associated protein SET. *Eur J Biochem*. 2001;268(5):1340-1351.

Moreno CS, Park S, Nelson K, et al. WD40 repeat proteins striatin and S/G(2) nuclear autoantigen are members of a novel family of calmodulin-binding proteins that associate with protein phosphatase 2A. *J Biol Chem*. 2000;275(8):5257-5263.

Moskalenko S, Henry DO, Rosse C, Mirey G, Camonis JH, White MA. The exocyst is a Ral effector complex. *Nat Cell Biol*. 2002;4(1):66-72.

Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev*. 2004;18(2):115-136.

Mullally A, Ebert BL. NF1 inactivation revs up Ras in adult acute myelogenous leukemia. *Clin Cancer Res*. 2010;16(16):4074-4076.

Mumby M. PP2A: unveiling a reluctant tumor suppressor. *Cell*. 2007;130(1):21-24.

Murata K, Wu J, Brautigan DL. B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. *Proc Natl Acad Sci U S A*. 1997;94(20):10624-10629.

Neviani P, Santhanam R, Oaks JJ, et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest*. 2007;117(9):2408-2421.

Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell*. 2005;8(5):355-368.

Niimi H, Harada H, Harada Y, et al. Hyperactivation of RAS signaling pathway in myelodysplastic syndrome with AML1/RUNX1point mutations. *Leukemia*. 2006;20(4):635-644.

Nolan C, Novoa WB, Krebs EG, Fischer EH. Further Studies on the Site Phosphorylated in the Phosphorylase B to a Reaction. *Biochemistry*. 1964;3:542-551.

Nucifora G, Laricchia-Robbio L, Senyuk V. EVI1 and hematopoietic disorders: history and perspectives. *Gene*. 2006;368:1-11.

O'Connell RM, Rao DS, Chaudhuri AA, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med*. 2008;205(3):585-594.

Odero MD, Vizmanos JL, Roman JP, et al. A novel gene, MDS2, is fused to ETV6/TEL in a t(1;12)(p36.1;p13) in a patient with myelodysplastic syndrome. *Genes Chromosomes Cancer*. 2002;35(1):11-19.

Ogris E, Du X, Nelson KC, et al. A protein phosphatase methylesterase (PME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A. *J Biol Chem*. 1999;274(20):14382-14391.

Ohta Y, Suzuki N, Nakamura S, Hartwig JH, Stossel TP. The small GTPase RalA targets filamin to induce filopodia. *Proc Natl Acad Sci U S A*. 1999;96(5):2122-2128.

Okamoto K, Li H, Jensen MR, et al. Cyclin G recruits PP2A to dephosphorylate Mdm2. *Mol Cell*. 2002;9(4):761-771.

Ory S, Zhou M, Conrads TP, Veenstra TD, Morrison DK. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr Biol*. 2003;13(16):1356-1364.

Ott MG, Schmidt M, Schwarzwaelder K, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med*. 2006;12(4):401-409.

Pallas DC, Shahrik LK, Martin BL, et al. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. *Cell*. 1990;60(1):167-176.

Parkin B, Ouillette P, Wang Y, et al. NF1 inactivation in adult acute myelogenous leukemia. *Clin Cancer Res*. 2010;16(16):4135-4147.

Paulsson K, Heidenblad M, Strombeck B, et al. High-resolution genome-wide array-based comparative genome hybridization reveals cryptic chromosome

changes in AML and MDS cases with trisomy 8 as the sole cytogenetic aberration. *Leukemia*. 2006;20(5):840-846.

Polakis P. Wnt signaling and cancer. *Genes Dev*. 2000;14(15):1837-1851.

Qu W, Li W, Wei L, Xing L, Wang X, Yu J. CIP2A is overexpressed in esophageal squamous cell carcinoma. *Med Oncol*. 2010. [Epub ahead of print].

Quintas-Cardama A, Manshouri T, Estrov Z, et al. Preclinical characterization of atiprimod, a novel JAK2 AND JAK3 inhibitor. *Invest New Drugs*. 2010. [Epub ahead of print].

Quintas-Cardama A, Vaddi K, Liu P, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood*. 2010;115(15):3109-3117.

Raghavan M, Lillington DM, Skoulakis S, et al. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. *Cancer Res*. 2005;65(2):375-378.

Roberts KG, Smith AM, McDougall F, et al. Essential requirement for PP2A inhibition by the oncogenic receptor c-KIT suggests PP2A reactivation as a strategy to treat c-KIT+ cancers. *Cancer Res*. 2010;70(13):5438-5447.

Rodriguez-Viciana P, Collins C, Fried M. Polyoma and SV40 proteins differentially regulate PP2A to activate distinct cellular signaling pathways involved in growth control. *Proc Natl Acad Sci U S A*. 2006;103(51):19290-19295.

Rosati R, La Starza R, Barba G, et al. Cryptic chromosome 9q34 deletion generates TAF-Ialpha/CAN and TAF-Ibeta/CAN fusion transcripts in acute myeloid leukemia. *Haematologica*. 2007;92(2):232-235.

Rowe JM. Optimal induction and post-remission therapy for AML in first remission. *Hematology Am Soc Hematol Educ Program*. 2009:396-405.

Rucker FG, Bullinger L, Schwaenen C, et al. Disclosure of candidate genes in acute myeloid leukemia with complex karyotypes using microarray-based molecular characterization. *J Clin Oncol*. 2006;24(24):3887-3894.

Ruediger R, Fields K, Walter G. Binding specificity of protein phosphatase 2A core enzyme for regulatory B subunits and T antigens. *J Virol*. 1999;73(1):839-842.

Ruediger R, Pham HT, Walter G. Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. *Oncogene*. 2001;20(15):1892-1899.

Ruediger R, Pham HT, Walter G. Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. *Oncogene*. 2001;20(1):10-15.

Ruvolo PP, Clark W, Mumby M, Gao F, May WS. A functional role for the B56 alpha-subunit of protein phosphatase 2A in ceramide-mediated regulation of Bcl2 phosphorylation status and function. *J Biol Chem*. 2002;277(25):22847-22852.

Sablina AA, Chen W, Arroyo JD, et al. The tumor suppressor PP2A Abeta regulates the RalA GTPase. *Cell*. 2007;129(5):969-982.

Sablina AA, Hahn WC. The role of PP2A A subunits in tumor suppression. *Cell Adh Migr*. 2007;1(3):140-141.

Sablina AA, Hector M, Colpaert N, Hahn WC. Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Res*. 2010;70(24):10474-10484.

Samanta AK, Chakraborty SN, Wang Y, et al. Jak2 inhibition deactivates Lyn kinase through the SET-PP2A-SHP1 pathway, causing apoptosis in drug-resistant cells from chronic myelogenous leukemia patients. *Oncogene*. 2009;28(14):1669-1681.

Sanz MA, Grimwade D, Tallman MS, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood*. 2009;113(9):1875-1891.

Sanz MA, Montesinos P, Vellenga E, et al. Risk-adapted treatment of acute promyelocytic leukemia with all-trans retinoic acid and anthracycline monochemotherapy: long-term outcome of the LPA 99 multicenter study by the PETHEMA Group. *Blood*. 2008;112(8):3130-3134.

Sargin B, Choudhary C, Crosetto N, et al. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. *Blood*. 2007;110(3):1004-1012.

Saydam G, Aydin HH, Sahin F, et al. Involvement of protein phosphatase 2A in interferon-alpha-2b-induced apoptosis in K562 human chronic myelogenous leukaemia cells. *Leuk Res*. 2003;27(8):709-717.

Schiffman JD, Wang Y, McPherson LA, et al. Molecular inversion probes reveal patterns of 9p21 deletion and copy number aberrations in childhood leukemia. *Cancer Genet Cytogenet*. 2009;193(1):9-18.

Schoch C, Kohlmann A, Schnittger S, et al. Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. *Proc Natl Acad Sci U S A*. 2002;99(15):10008-10013.

Schoch C, Schnittger S, Klaus M, et al. AML with 11q23/MLL abnormalities as defined by the WHO classification: incidence, partner chromosomes, FAB subtype, age distribution, and prognostic impact in an unselected series of 1897 cytogenetically analyzed AML cases. *Blood*. 2003;102(7):2395-402.

Schonthal AH. Role of serine/threonine protein phosphatase 2A in cancer. *Cancer Lett*. 2001;170(1):1-13.

Schwind S, Maharry K, Radmacher MD, et al. Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2010;28(36):5257-5264.

Sears R, Leone G, DeGregori J, Nevins JR. Ras enhances Myc protein stability. *Mol Cell*. 1999;3(2):169-179.

Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev*. 2000;14(19):2501-2514.

Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer*. 2004;4(12):937-947.

Seeling JM, Miller JR, Gil R, Moon RT, White R, Virshup DM. Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. *Science*. 1999;283(5410):2089-2091.

Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell*. 2001;104(1):119-130.

Shannon KM, O´Connell P, Martin GA, et al. Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *N Engl J Med*. 1994;330(9):597-601.

Shimabe M, Goyama S, Watanabe-Okochi N, et al. Pbx1 is a downstream target of Evi-1 in hematopoietic stem/progenitors and leukemic cells. *Oncogene*. 2009;28(49):4364-4374.

Shouse GP, Nobumori Y, Liu X. A B56gamma mutation in lung cancer disrupts the p53-dependent tumor-suppressor function of protein phosphatase 2A. *Oncogene*. 2010;29(27):3933-41.

Shtrichman R, Sharf R, Kleinberger T. Adenovirus E4orf4 protein interacts with both Balpha and B' subunits of protein phosphatase 2A, but E4orf4-induced apoptosis is mediated only by the interaction with Balpha. *Oncogene*. 2000;19(33):3757-3765.

Silverstein AM, Barrow CA, Davis AJ, Mumby MC. Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. *Proc Natl Acad Sci U S A*. 2002;99(7):4221-4226.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.

Sonoda Y, Kasahara T, Yamaguchi Y, Kuno K, Matsushima K, Mukaida N. Stimulation of interleukin-8 production by okadaic acid and vanadate in a human promyelocyte cell line, an HL-60 subline. Possible role of mitogen-activated protein kinase on the okadaic acid-induced NF-kappaB activation. *J Biol Chem*. 1997;272(24):15366-15372.

Sonoda Y, Yamamoto D, Sakurai S, et al. FTY720, a novel immunosuppressive agent, induces apoptosis in human glioma cells. *Biochem Biophys Res Commun*. 2001;281(2):282-288.

Sontag E, Fedorov S, Kamibayashi C, Robbins D, Cobb M, Mumby M. The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. *Cell*. 1993;75(5):887-897.

Soo Hoo L, Zhang JY, Chan EK. Cloning and characterization of a novel 90 kDa 'companion' auto-antigen of p62 overexpressed in cancer. *Oncogene*. 2002;21(32):5006-5015.

Starczynowski DT, Morin R, McPherson A, et al. Genome-wide identification of human microRNAs located in leukemia-associated genomic alterations. *Blood*. 2011;117(2):595-607.

Stevens I, Janssens V, Martens E, Dilworth S, Goris J, Van Hoof C. Identification and characterization of B"-subunits of protein phosphatase 2 A in Xenopus laevis oocytes and adult tissues. *Eur J Biochem*. 2003;270(2):376-387.

Stokoe D, McCormick F. Activation of c-Raf-1 by Ras and Src through different mechanisms: activation in vivo and in vitro. *EMBO J*. 1997;16(9):2384-2396.

Stone RM, O'Donnell MR, Sekeres MA. Acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*. 2004:98-117.

Stone SR, Hofsteenge J, Hemmings BA. Molecular cloning of cDNAs encoding two isoforms of the catalytic subunit of protein phosphatase 2A. *Biochemistry*. 1987;26(23):7215-7220.

Strack S, Chang D, Zaucha JA, Colbran RJ, Wadzinski BE. Cloning and characterization of B delta, a novel regulatory subunit of protein phosphatase 2A. *FEBS Lett*. 1999;460(3):462-466.

Strack S, Cribbs JT, Gomez L. Critical role for protein phosphatase 2A heterotrimers in mammalian cell survival. *J Biol Chem*. 2004;279(46):47732-47739.

Suela J, Alvarez S, Cifuentes F, et al. DNA profiling analysis of 100 consecutive de novo acute myeloid leukemia cases reveals patterns of genomic instability that affect all cytogenetic risk groups. *Leukemia*. 2007;21(6):1224-1231.

Suganuma M, Fujiki H, Furuya-Suguri H, et al. Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin. *Cancer Res*. 1990;50(12):3521-3525.

Suganuma M, Fujiki H, Suguri H, et al. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc Natl Acad Sci U S A*. 1988;85(6):1768-1771.

Takagi Y, Futamura M, Yamaguchi K, Aoki S, Takahashi T, Saji S. Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. *Gut*. 2000;47(2):268-271.

Tamaki M, Goi T, Hirono Y, Katayama K, Yamaguchi A. PPP2R1B gene alterations inhibit interaction of PP2A-Abeta and PP2A-C proteins in colorectal cancers. *Oncol Rep*. 2004;11(3):655-659.

Tehrani MA, Mumby MC, Kamibayashi C. Identification of a novel protein phosphatase 2A regulatory subunit highly expressed in muscle. *J Biol Chem*. 1996;271(9):5164-5170.

ten Klooster JP, Leeuwen I, Scheres N, Anthony EC, Hordijk PL. Rac1-induced cell migration requires membrane recruitment of the nuclear oncogene SET. *EMBO J*. 2007;26(2):336-345.

Tomasson MH, Xiang Z, Walgren R, et al. Somatic mutations and germline sequence variants in the expressed tyrosine kinase genes of patients with de novo acute myeloid leukemia. *Blood*. 2008;111(9):4797-4808.

Vaarala MH, Vaisanen MR, Ristimaki A. CIP2A expression is increased in prostate cancer. *J Exp Clin Cancer Res*. 2010;29:136.

Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1617-1628.

Vardiman JW. The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms. *Chem Biol Interact*. 2010;184(1-2):16-20.

von Lindern M, van Baal S, Wiegant J, Raap A, Hagemeijer A, Grosveld G. Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. *Mol Cell Biol*. 1992;12(8):3346-3355.

Voorhoeve PM, Hijmans EM, Bernards R. Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein. *Oncogene*. 1999;18(2):515-524.

Waldman SA, Terzic A. A study of microRNAs in silico and in vivo: diagnostic and therapeutic applications in cancer. *FEBS J*. 2009;276(8):2157-2164.

Walter MJ, Payton JE, Ries RE, et al. Acquired copy number alterations in adult acute myeloid leukemia genomes. *Proc Natl Acad Sci U S A*. 2009;106(31):12950-5.

Wang GL, Iakova P, Wilde M, Awad S, Timchenko NA. Liver tumors escape negative control of proliferation via PI3K/Akt-mediated block of C/EBP alpha growth inhibitory activity. *Genes Dev*. 2004;18(8):912-925.

Wang J, Li W, Li L, Yu X, Jia J, Chen C. CIP2A is over-expressed in acute myeloid leukaemia and associated with HL60 cells proliferation and differentiation. *Int J Lab Hematol*. 2011. [Epub ahead of print].

Wang SS, Esplin ED, Li JL, et al. Alterations of the PPP2R1B gene in human lung and colon cancer. *Science*. 1998;282(5387):284-287.

Wassarman DA, Solomon NM, Chang HC, Karim FD, Therrien M, Rubin GM. Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in Drosophila. *Genes Dev*. 1996;10(3):272-278.

Weksberg R, Hughes S, Moldovan L, Bassett AS, Chow EW, Squire JA. A method for accurate detection of genomic microdeletions using real-time quantitative PCR. *BMC Genomics*. 2005;6:180.

Westbrook CA, Hooberman AL, Spino C, et al. Clinical significance of the BCR-ABL fusion gene in adult acute lymphoblastic leukemia: a Cancer and Leukemia Group B Study (8762). *Blood*. 1992;80(12):2983-2990.

Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med*. 2008;14(4):152-160.

Westermarck J, Holmstrom T, Ahonen M, Eriksson JE, Kahari VM. Enhancement of fibroblast collagenase-1 (MMP-1) gene expression by tumor promoter okadaic acid is mediated by stress-activated protein kinases Jun N-terminal kinase and p38. *Matrix Biol*. 1998;17(8-9):547-557.

Xing Y, Xu Y, Chen Y, et al. Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell*. 2006;127(2):341-353.

Xu Y, Xing Y, Chen Y, et al. Structure of the protein phosphatase 2A holoenzyme. *Cell*. 2006;127(6):1239-1251.

Yan Z, Fedorov SA, Mumby MC, Williams RS. PR48, a novel regulatory subunit of protein phosphatase 2A, interacts with Cdc6 and modulates DNA replication in human cells. *Mol Cell Biol*. 2000;20(3):1021-1029.

Yang J, Wu J, Tan C, Klein PS. PP2A:B56epsilon is required for Wnt/beta-catenin signaling during embryonic development. *Development*. 2003;130(23):5569-5578.

Yasui H, Hideshima T, Raje N, et al. FTY720 induces apoptosis in multiple myeloma cells and overcomes drug resistance. *Cancer Res*. 2005;65(16):7478-7484.

Yendamuri S, Calin GA. The role of microRNA in human leukemia: a review. *Leukemia*. 2009;23(7):1257-1263.

Yokoyama N, Reich NC, Miller WT. Determinants for the interaction between Janus kinase 2 and protein phosphatase 2A. *Arch Biochem Biophys*. 2003;417(1):87-95.

Yokoyama N, Reich NC, Miller WT. Involvement of protein phosphatase 2A in the interleukin-3-stimulated Jak2-Stat5 signaling pathway. *J Interferon Cytokine Res*. 2001;21(6):369-378.

Zaidi SZ, Owaidah T, Al Sharif F, Ahmed SY, Chaudhri N, Aljurf M. The challenge of risk stratification in acute myeloid leukemia with normal karyotype. *Hematol Oncol Stem Cell Ther*. 2008;1(3):141-158.

Zeng W, Wharton KA, Jr., Mack JA, et al. naked cuticle encodes an inducible antagonist of Wnt signalling. *Nature*. 2000;403(6771):789-795.

Zhang Y, Taylor BR, Shannon K, Clapp DW. Quantitative effects of Nf1 inactivation on in vivo hematopoiesis. *J Clin Invest*. 2001;108(5):709-715.

Zhou J, Pham HT, Ruediger R, Walter G. Characterization of the Aalpha and Abeta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. *Biochem J*. 2003;369(Pt 2):387-398.

Ziogas A, Moelling K, Radziwill G. CNK1 is a scaffold protein that regulates Src-mediated Raf-1 activation. *J Biol Chem*. 2005;280(25):24205-24211.

Zolnierowicz S, Csortos C, Bondor J, Verin A, Mumby MC, DePaoli-Roach AA. Diversity in the regulatory B-subunits of protein phosphatase 2A: identification of a novel isoform highly expressed in brain. *Biochemistry*. 1994;33(39):11858-11867.

ANEXOS

blood

2010 115: 615-625
Prepublished online November 16, 2009;
doi:10.1182/blood-2009-06-227363

SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia

Ion Cristóbal, Francisco J. Blanco, Laura Garcia-Orti, Nerea Marcotegui, Carmen Vicente, José Rifon, Francisco J. Novo, Eva Bandres, María J. Calasanz, Carmelo Bernabeu and María D. Odero

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia

Ion Cristóbal,[1,2] Francisco J. Blanco,[3] Laura Garcia-Orti,[1] Nerea Marcotegui,[1] Carmen Vicente,[1,2] José Rifon,[4] Francisco J. Novo,[2] Eva Bandres,[1] María J. Calasanz,[2] Carmelo Bernabeu,[3] and María D. Odero[1,2]

[1]Division of Oncology, Center for Applied Medical Research, and [2]Department of Genetics, University of Navarra, Pamplona; [3]Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), and CIBER de Enfermedades Raras (CIBERER), Madrid; and [4]Clínica Universidad de Navarra, University of Navarra, Pamplona, Spain

Acute myeloid leukemias (AMLs) result from multiple genetic alterations in hematopoietic stem cells. We describe a novel t(12;18)(p13;q12) involving *ETV6* in a patient with AML. The translocation resulted in overexpression of *SETBP1* (18q12), located close to the breakpoint. Overexpression of *SETBP1* through retroviral insertion has been reported to confer growth advantage in hematopoietic progenitor cells. We show that *SETBP1* overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein and leading to the formation of a SETBP1–SET-PP2A complex that results in PP2A inhibition, promoting proliferation of the leukemic cells. The prevalence of *SETBP1* overexpression in AML at diagnosis (n = 192) was 27.6% and was associated with unfavorable cytogenetic prognostic group, monosomy 7, and *EVI1* overexpression ($P < .01$). Patients with *SETBP1* overexpression had a significantly shorter overall survival, and the prognosis impact was remarkably poor in patients older than 60 years in both overall survival ($P = .015$) and event-free survival ($P = .015$). In summary, our data show a novel leukemogenic mechanism through *SETBP1* overexpression; moreover, multivariate analysis confirms the negative prognostic impact of *SETBP1* overexpression in AML, especially in elderly patients, where it could be used as a predictive factor in any future clinical trials with PP2A activators. (Blood. 2010;115: 615-625)

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal malignancy that predominantly affects middle-aged and elderly adults. The disease is characterized by a differentiation block in early progenitors, which leads to the accumulation of immature cells in bone marrow (BM) and peripheral blood. In recent years, several genetic markers with prognostic impact in AML have been identified, leading to a better understanding of the biology of this disease and, in some cases, providing targets for molecular therapies.[1] Cytogenetic aberrations have been reported as the most important prognostic factors for survival and response to therapy in AML, allowing the identification of molecular markers that have greatly advanced our understanding of leukemogenesis[2]; nevertheless, the nature of alterations responsible for initiation or progression of the disease is mostly unknown. Recent attempts to identify initiating or progression mutations by extensively resequencing tyrosine kinase genes,[3,4] expression profiling studies,[5-7] array-based comparative genomic hybridization and/or single nucleotide polymorphism,[8-11] and even by unbiased whole-genome sequencing,[12] confirm that AML results from multiple genetic alterations in hematopoietic stem cells, and suggest that we have not yet discovered most of the relevant aberrations that contribute to the pathogenesis of this disease.

The *ETV6* gene (12p13) encodes a transcription factor frequently rearranged in both myeloid and lymphoid leukemias. Translocation breakpoints are distributed throughout the gene, and *ETV6* contributes to the pathogenesis of leukemia by diverse molecular mechanisms that are only partially understood. In most cases, the translocations result in the generation of in-frame fusion genes between different domains of *ETV6* and partner genes encoding either kinases or transcription factors.[13] However, in some cases involving the 5′ end of *ETV6*, functionally significant fusions could not be detected and a different leukemogenic mechanism has been described: the deregulation of the expression of oncogenes located close to the breakpoints.[13,14] This molecular mechanism, which has been described mainly in lymphoid leukemias and lymphomas, is an uncommon mechanism in myeloid leukemias, although some examples have been reported.[13,15]

Here, we describe a novel leukemogenic mechanism in a patient with AML and a t(12;18)(p13;q12) involving *ETV6*. The translocation resulted in overexpression of *SETBP1* (18q12), located close to the breakpoint. We show that *SETBP1* overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein and leading to the formation of a SETBP1–SET-PP2A complex that results in PP2A inhibition and therefore promotes the proliferation of leukemic cells. Moreover, we show that *SETBP1* overexpression is a recurrent molecular event associated with a significantly shorter overall survival (OS) in AML, especially in elderly patients.

Submitted June 11, 2009; accepted October 21, 2009. Prepublished online as *Blood* First Edition paper, November 16, 2009; DOI 10.1182/blood-2009-06-227363.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Methods

Case reports

A 76-year-old white man was diagnosed with myelodysplastic syndrome. Disease evaluation of the patient 3 years after the diagnosis showed anorexia, perspiration, and loss of 7 kg. Laboratory findings at this moment were: hemoglobin 10.9 g/dL, white blood cell count 38.2×10^9/L with 55% blasts, and platelet count 251×10^9/L; BM aspirate was hypercellular, showing 80% blasts. Flow cytometry showed positivity for CD34, CD13, CD33, CD11b, and HLA-DR. The patient was diagnosed as AML-M5. Karyotype showed 2 clones: 46,XY,t(12,18)(p13;q12)[77%]/47,idem,+19[23%]. The patient received standard induction chemotherapy for 2 months and had partial remission at the next evaluation. Laboratory findings showed hemoglobin 9.9 g/dL, white blood cell count 25.5×10^9/L with 32% blasts, and 260×10^9/L platelets. Karyotype at this time was: 46,XY,t(12,18)(p13;q12)[24%]/46,XY[76%]. The patient relapsed 2 months later and eventually died. Karyotype at relapse showed the 2 clones detected at diagnosis: 46,XY,t(12,18)(p13;q12)[32%]/47,idem,+19[68%]. All samples were taken anonymously, and the study was approved by the Institutional Committee of the University of Navarra.

FISH

Bacterial artificial chromosomes obtained from the Roswell Park Cancer Institute (Buffalo, NY) were used to map the breakpoints in the patient samples. The order of the probes is centromere-418C2-96B19-434C1-telomere (12p13), and centromere-840B16-937P23-252G8-941F5-telomere (18q12). Probes were labeled with SpectrumGreen-dUTP or SpectrumOrange-dUTP. Centromeric probes for chromosomes 12 and 18 were also used in fluorescence in situ hybridization (FISH) experiments. FISH was performed as previously described.[14]

RACE

3′- and 5′-rapid amplification of cDNA ends (RACE)–polymerase chain reaction (PCR) were performed from total RNA using the GeneRacer kit (Invitrogen). 3′RACE-PCR (94°C 30 seconds, 72°C 2 minutes 30 seconds, 5 cycles; 94°C 30 seconds, 70°C 2 minutes 30 seconds, 5 cycles; 94°C 30 seconds, 64°C 30 seconds, 68°C 2 minutes 30 seconds, 25 cycles) was performed using the primer ETV6-EX1F (exon 1) and a reverse primer from the kit. Nested PCR was performed under the same conditions with ETV6-EX1Fn (exon 1). Products were cloned and sequenced. For 5′RACE, the same PCR conditions were used, with primers ETV6-EX3R and ETV6-EX3Rn (exon 3; supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Nucleic acid isolation and RT-PCR

Total DNA was isolated using the QIAmp DNA minikit, and total RNA using the RNeasy minikit (QIAGEN). cDNA was synthesized with SuperScriptIII Reverse Transcriptase (Invitrogen). Reverse-transcribed (RT)–PCR reactions were carried out with Ampli-Taq Gold DNA Polymerase (Applied Biosystems) after optimizing cycling conditions for each primer pair. To confirm the presence of the fusion products, RT-PCR reactions were performed on patient RNA with primers ETV6-EX1F2, and CR18-B and CR18-E (supplemental Table 1). Products were cloned and sequenced.

Real-time RT-PCR

Quantification of the expression of *SETBP1*, *SET*, and *ETV6* was performed using TaqMan Gene Expression Assays (Applied Biosystems) specific for each gene. *GAPDH* was used as internal control. A gene was considered overexpressed if its expression value was higher than the cut-off value established for each gene (mean ± 5 SD), defined by the analysis of 10 normal BM samples.

Cell culture and transfection

HEL, K562, 32Dcl3, and HEK293 cell lines were grown at 37°C in a 5% CO_2 atmosphere. HEK293 cells were maintained in Dulbecco's minimum essential medium, HEL and K562 in RPMI-1640 (Invitrogen), and 32Dcl3 in RPMI 1640 + 10% conditioned medium of cell line WEHI-3B (DSM ACC 26). Media were supplemented with 10% fetal bovine serum, penicillin G (100 U/mL), and streptomycin (0.1 mg/mL). For transfection experiments, HEK293 cells were seeded into 10-cm dishes and transfected with 60 μL of Lipofectamine2000 (Invitrogen) and 12 μg of the plasmids expressing SETBP1(SKI homologous region [SHR])–V5, SETBP1-GFP, SETBP1(SETBD)–GFP, SET-GFP, or empty vector as control. HEL and 32Dcl3 cells were seeded in culture flasks and transfected using the Nucleofector System (solution V; protocol X-005 for HEL, and E-032 for 32Dcl3; Amaxa), with 4 μg of plasmidic vectors or 75nM *SET* siRNA pool designed and synthesized by Dharmacon RNA Technologies.

Plasmids

Human *SET* cDNA was obtained by RT-PCR from K562 RNA using an upstream primer containing an *Eco*RI site followed by the first 19 nucleotides of *SET* cDNA, and a downstream primer containing the last 21 nucleotides of *SET* linked to a *Bam*HI site. The *Eco*RI/*Bam*HI digested PCR product was subcloned into the pEGFP-C2 vector leading to the pEGFPC2-SET construct. Human *SETBP1* cDNA was obtained by RT-PCR from peripheral blood and subcloned into the vector pEGFP-C2 through *Xho*I/SalI sites, resulting in the pEGFPC2-SETBP1 construct. The region from amino acids 1167 to the end of SETBP1 was obtained by digestion from the pEGFPC2-SETBP1 construct and subcloned into the vector pEGFP-C2 through *Hin*dIII/SacII sites, resulting in the pEGFPC2-SETBP1(SETBD) construct. The region from amino acids 449 to amino acids 857 of SETBP1 was obtained by PCR from the pEGFPC2-SETBP1 and subcloned into the pcDNA3.1V5/His vector, leading to the pcDNA3.1V5/His-SETBP1(SHR). All cloning procedures were verified by sequencing.

Immunoprecipitation and Western blotting

Cells were lysed in 100 μL of lysis buffer containing 1% Triton X-100 and protease inhibitors (Complete Mini; Roche Diagnostics). After incubation on ice (30 minutes), protein extracts were clarified (12 000*g*, 15 minutes, 4°C), denatured, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. Antibodies used were goat polyclonal anti-SET (Santa Cruz Biotechnology), mouse monoclonal anti-PP2A (clone 1D6, Upstate), rabbit monoclonal anti-PP2A^{Y307} (Epitomics), rabbit polyclonal anti-GFP (Santa Cruz Biotechnology), and mouse monoclonal anti-βtubulin (Sigma-Aldrich). For immunoprecipitation, lysates were precleared (1 hour, 4°C) on a rotating wheel and immunoprecipitated with antibody-bound protein G-Sepharose (8 hours, 4°C). After washings, immunoprecipitations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit; GE Healthcare).

Proliferation assay, viability, and total cell counts

Cell proliferation was measured in triplicate wells by 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Cell viability and total counts were measured in triplicate with the Nucleocounter (Biogen), and the results confirmed by Trypan Blue method.

PP2A assays

PP2A assays were performed with cell lysates (50 μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described,[16] except that we added the protease inhibitor cocktail Complete (Roche Diagnostics) to the protein extraction mix.

Statistical analysis

Statistical analyses were performed using SPSS 15 for windows (SPSS Inc). OS was defined as the time from diagnosis to death of any cause or end of follow-up. Disease-free survival (DFS) was defined as the time from complete remission until relapse or death. Event-free survival (EFS) was defined as the time from diagnosis until first event, in which failure to achieve complete remission, relapse, death, or end of follow-up were considered events. OS, DFS, and EFS were determined according to the Kaplan-Meier method and survival comparisons were done with the log-rank test if proportional hazard assumption was fulfilled, and Breslow otherwise. The Cox proportional hazards model was used to assess patient outcome for patient-stratified groups of age. The hazard model was adjusted taking into consideration relevant parameters that included cytogenetic group, complete remission, and *SETBP1* overexpression. Good risk and intermediate risk were analyzed together because of the small number of patients with good-risk cytogenetics. A P value less than .05 was considered statistically significant.

Bioinformatics analysis of the *SETBP1* proximal promoter

Analysis of the proximal promoter of *SETBP1* was performed with MotifScanner,[17-19] which scans DNA sequences with precompiled motif models. This algorithm assumes that motifs are hidden in a noisy background sequence represented by a higher-order Markov model. The motif models of transcriptional factors were obtained from the public version of Jaspar and Transfac databases. The proximal promoter of *SETBP1* was defined as 3000 bp upstream the transcription start site and was extracted from Ensembl database release 53.

Results

SETBP1 (18q21) is overexpressed in a patient with a translocation t(12;18) involving *ETV6*

Karyotype at diagnosis of a patient with AML-M5 secondary to myelodysplastic syndrome was 46,XY,t(12,18)(p13;q12)/47,idem,+19. FISH showed that the breakpoint on 12p13 was located between exons 2 and 3 of *ETV6* (supplemental Figure 1). To identify the fusion partner of *ETV6*, RACE-PCR experiments were performed on RNA from BM of the patient. 3′RACE-PCR identified 2 clones containing *ETV6* exon 2 followed by sequences that overlapped with the human cDNA clone BC051727 (Mammalian Gene Collection Program Team) located in chromosome 18 (Figure 1). Alignment of these sequences to the human genome did not reveal the presence of a known gene, but the novel sequences were split into segments that were flanked by consensus splice donor and acceptor sites. This indicated that these sequences could be part of a novel gene, whose exact identity and complete cDNA sequence were not further analyzed. Because the complete transcript is currently unknown, the different exons identified in this sequence were arbitrarily named as follows: fusion transcript 1, *ETV6*-exon-1 + *ETV6*-exon-2 + exon-“a” + exon-“b”; fusion transcript 2, *ETV6*-exon-1 + *ETV6*-exon-2 + exon-“c” + exon-“d” + exon-“e.” Stop codons were found in the 3 different reading frames for exons

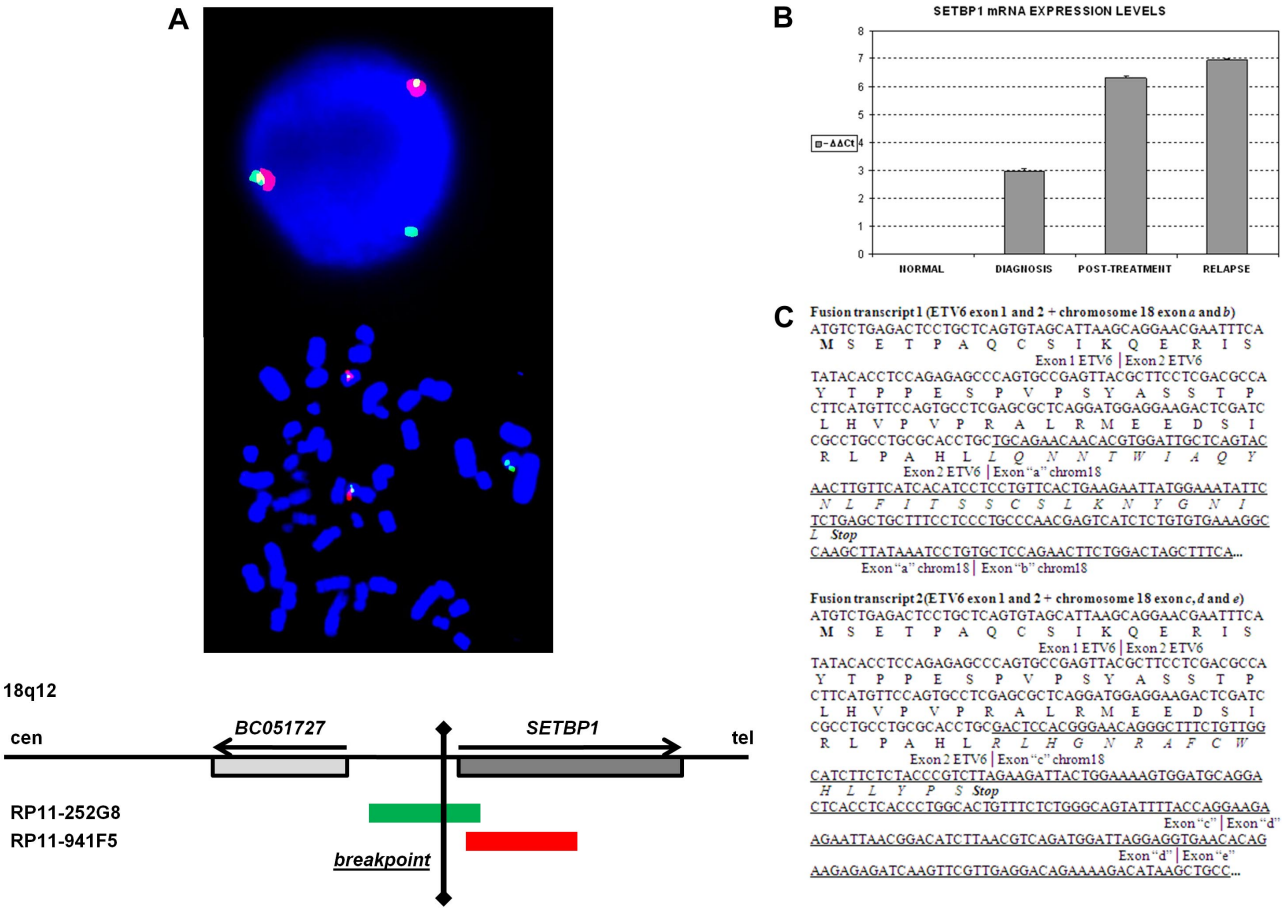


Figure 1. Genetic characterization of a patient with AML-M5 and a t(12;18)(p13;q12) involving *ETV6*. (A) FISH analysis indicating the breakpoint on 18q12: probe RP11-252G8 (green) splits and hybridizes in both der(18) and der(12). (B) Analysis by quantitative RT-PCR of the *SETBP1* expression in samples of the patient at diagnosis, posttreatment control, and relapse. (C) RACE results showing the 2 different fusion transcripts detected. Sequences from *ETV6* are in italics; the putative exons from chromosome 18 are underlined.

"b" and "e," indicating that these exons are part of a noncoding transcript. The presence of these 2 fusions was confirmed by RT-PCR, cloned, and sequenced (data not shown). Fusion transcripts predicted, in both cases, truncated proteins resulting from the presence of premature stop codons (Figure 1). Besides, the predicted new proteins showed no homology with any known proteins. In these novel sequences, we found no microRNAs that could be deregulated as a consequence of the translocation. 5′RACE-PCR failed to find any reciprocal fusion transcript. Taken together, these data suggest that no functionally significant fusion transcripts were generated by the translocation.

To confirm the position of the breakpoint on chromosome 18, bacterial artificial chromosomes located at 18q12 were used as probes in FISH experiments. Analysis on BM cells of the patient showed that one signal hybridized to the normal chromosome 18, and the other split and hybridized to both der(18) and der(12) (Figure 1). FISH showed that the breakpoint was located 5′ and close to the *SETBP1* gene. Activation of *SETBP1* expression by retroviral integration in hematopoietic progenitor cells has been reported to confer a growth advantage leading to clonal expansion.[20] Knowing that *SETBP1* was close to the breakpoint in the t(12;18)(p13;q12) and taking into account that ectopic expression of oncogenes is a mechanism involved in leukemia, we analyzed the expression of *SETBP1* by real-time PCR (quantitative RT-PCR) in this patient, and we found that *SETBP1* was overexpressed at diagnosis and in the posttreatment samples (Figure 1).

Ectopic expression of SETBP1 leads to increased full-length SET protein levels

SETBP1 has been reported to specifically interact with the protein SET,[21] a potent inhibitor of protein phosphatase 2A (PP2A). To assess whether the ectopic expression of SETBP1 affects SET protein levels, HEK293 cells were transiently transfected with SETBP1-GFP. We found that levels of the 39-kDa full-length form of SET were strongly induced; moreover, an important decrease in the levels of low molecular weight forms of SET was observed. As controls, ectopic expression of the SETBP1-GFP protein was detected by anti-GFP (Figure 2A) and quantitative RT-PCR (supplemental Figure 2A). Analysis by fluorescence microscopy of SETBP1-GFP–transfected HEK293 cells confirmed a predominantly nuclear location of SETBP1 (supplemental Figure 2B).

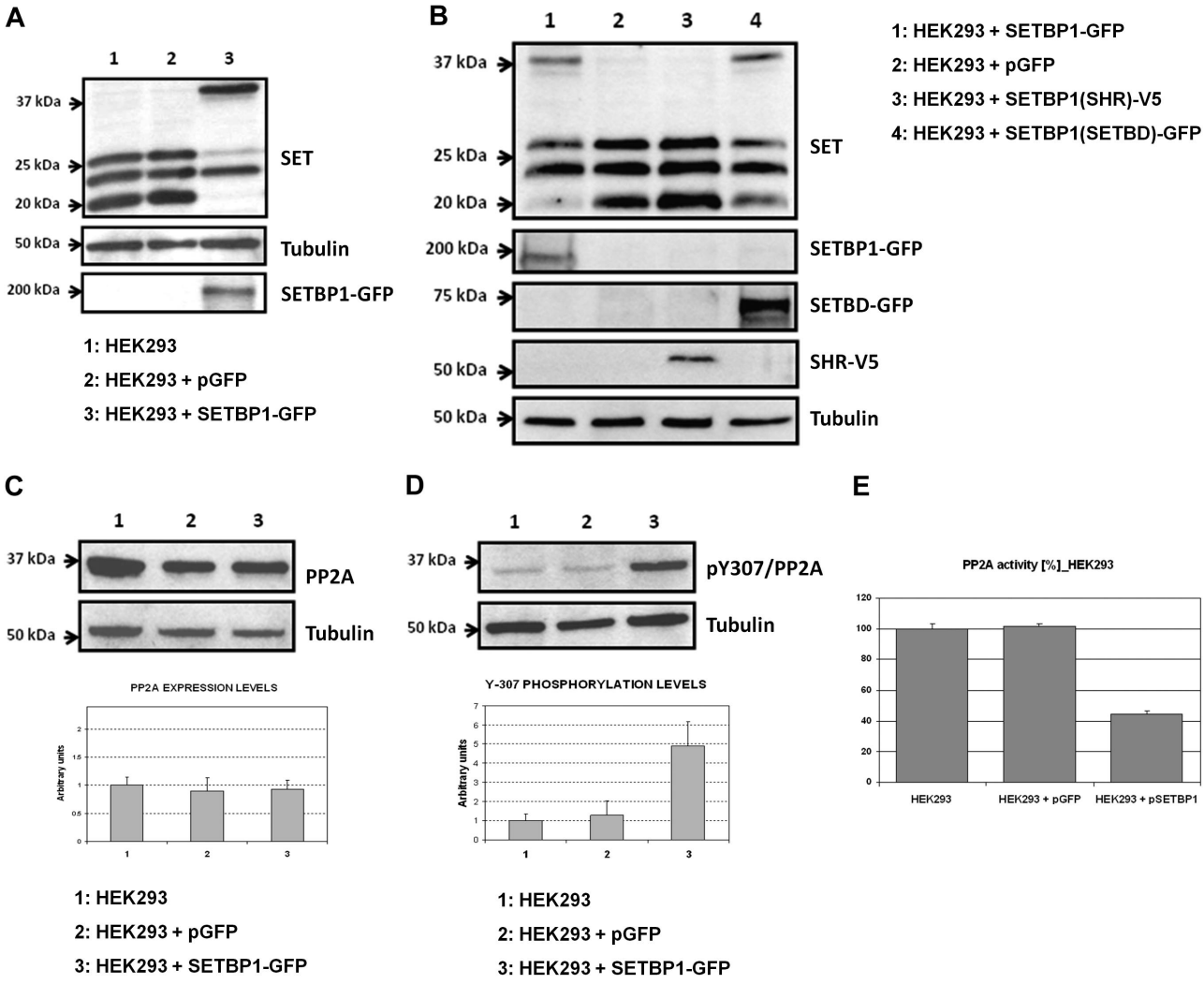


Figure 2. Ectopic expression of SETBP1 induces increased full-length SET levels and inhibits PP2Ac. (A) Western blot showing endogenous SET expression in HEK293 cells transfected with SETBP1-GFP, an empty vector as a control, or nontransfected cells. (B) Analysis by Western blot showing SET in HEK293 cells transfected with different regions of SETBP1. SHR indicates SKI homologous region; and SETBD, SET binding domain. (C) Western blot and densitometry analysis showing the effect of ectopic SETBP1 over PP2A expression in HEK293 cells. (D) Western blot and densitometry analysis showing the effect of ectopic SETBP1 over PP2A phosphorylation levels of the tyrosine-307. (E) PP2A phosphatase assay in HEK293 cells transfected or not with SETBP1.

To assess the origin of the proteins of different sizes detected by anti-SET antibodies, HEK293 cells were transfected with SET-GFP and harvested at different times. Full-length SET-GFP protein of approximately 67 kDa could be detected by Western blot 9 hours after transfection, and 2 additional bands of lower molecular weight appeared gradually, together with the full-length form (supplemental Figure 2C). Taking into account the molecular weight of GFP, these bands would correspond to the endogenous short SET forms (Figure 2A). Because SET-GFP is expressed from a cDNA sequence, and alternative splicing cannot take place in the absence of intronic sequences, these results suggest that the endogenous SET protein of 39 kDa is cleaved by proteases, resulting in truncated proteins with distinct sizes.

To analyze the region of SETBP1 that is critical to induce this effect, we transfected HEK293 cells with the vector pcDNA3.1-SETBP1(SHR)-V5, which expresses an internal region of SETBP1 that includes the SHR, and with the vector pEGFPC2-SETBP1(SETBD), expressing the carboxy-terminal region of SETBP1 that includes the SET binding domain (SETBD). We used HEK293 cells transfected with SETBP1-GFP as positive control, with the empty vector as negative control. We found similar results transfecting with SETBP1-GFP and with SETBD-GFP; however, no effects were observed with SETBP1(SHR)-V5, indicating that the region containing SETBD is critical (Figure 2B). Taken together, these results suggest that SETBP1 has a major effect on the 39-kDa form of SET, probably increasing its levels by impairing protease activity on the full-length SET protein.

SETBP1 inhibits PP2A activity through tyrosine phosphorylation of its catalytic subunit

We next investigated the effect of SETBP1 overexpression on the PP2A protein. Expression levels of the catalytic subunit of PP2A (PP2Ac) were not affected in HEK293 cells transfected with SETBP1-GFP, with respect to nontransfected cells (Figure 2C). However, an increase in the phosphorylation of tyrosine-307 was observed (Figure 2D). Of note, phosphorylation of tyrosine-307 is responsible for more than 90% of the phosphatase activity of this protein; indeed, it has been shown that PP2Ac is inactive when tyrosine-307 is phosphorylated.[22] These results suggest that ectopic expression of SETBP1 leads to a reduced PP2A activity, which was confirmed by a PP2A phosphatase assay (Figure 2E).

We next assessed whether the observations made in SETBP1-transfected cells could be confirmed in the patient samples. As a positive control for PP2A inactivation and SET overexpression, protein extracts from K562 cells were included.[23] The patient showed higher phosphorylation of tyrosine-307 of PP2Ac in both relapse and posttreatment samples, compared with normal donors. We also observed increased SET protein levels, with good correlation between protein and mRNA (Figure 3; supplemental Figure 3).

SETBP1 induces proliferation in HEL and 32Dcl3

To investigate the effect of SETBP1 on cell growth, the HEL cell line (AML-M6) was chosen as a cellular model. An increased proliferation in cells transfected with SETBP1 compared with mock-transfected cells was observed using the MTS assay (Figure 4A); furthermore, total cell counts and cell viability were determined by Nucleo Counter and confirmed with the Trypan Blue method (data not shown). These results confirmed the higher cell counts and better viability of cells transfected with SETBP1 compared with cells transfected with the empty vector. Similar results were obtained with the 32Dcl3 cell line (Figure 4A). In addition, we observed reduced PP2A activity in both HEL and 32Dcl3 cells transfected with SETBP1.

To confirm our hypothesis that SETBP1 inhibits PP2A through SET, SET was down-regulated by *SET*-specific siRNAs (supplemental Figure 4). We observed that the effects of SETBP1 on cell proliferation and PP2A activity were impaired in both HEL and 32Dcl3 cells after *SET* knockdown (Figure 4B).

SETBP1 forms a complex including SET and PP2A

It has been reported that SETBP1 interacts with SET and that the region of SET that binds to SETBP1 is different from the PP2A inhibitory region. Accordingly, it was postulated that SETBP1-SET heterodimers interact with PP2A.[21] However, no experimental evidence to confirm this hypothesis has been provided yet. To determine whether SET interacts simultaneously with SETBP1 and PP2A, coimmunoprecipitation assays were carried out in HEK293 cells, transfected or not with SETBP1-GFP. PP2A and SET coimmunoprecipitated with SETBP1, suggesting the existence of a SETBP1–SET-PP2A complex. Preclearing of SET using anti-SET antibodies bound to Sepharose-protein G inhibited the coimmunoprecipitation of PP2A and SET with SETBP1, indicating that PP2A interacts with the SETBP1-SET heterodimer through SET and that SETBP1 and PP2A do not directly interact with each other. Interestingly, we found that the coimmunoprecipitated PP2A was phosphorylated on its tyrosine-307 (Figure 5). Taken together, these results suggest that the SETBP1-SET heterodimer interacts with PP2A forming a SETBP1–SET-PP2A complex in which the presence of SET is critical.

Prevalence of SETBP1 overexpression in AML

To study the prevalence of *SETBP1* overexpression and its prognostic value in AML, we quantified the expression of *SETBP1* in a series of 192 patients with AML at diagnosis, correlated these values with the French-American-British classification, and cytogenetic and molecular markers, and studied the prognostic relevance of this aberration. Patient characteristics are presented in Table 1, and box-plots show the *SETBP1* expression levels in supplemental Figure 5. Although patients were treated with different schedules, all received regimens based on anthracycline and cytarabine as

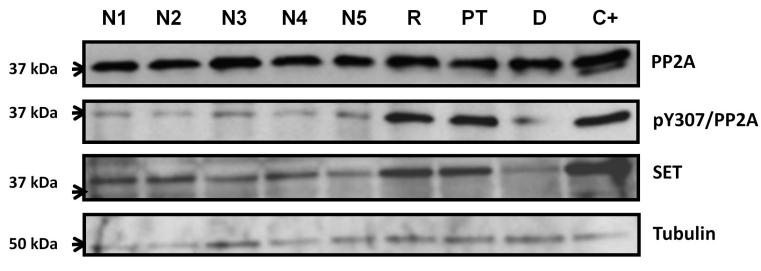


Figure 3. Comparison of the PP2A and SET protein levels by Western blot between patient samples and normal donors. Phosphorylation on tyrosine-307 of PP2A is also assessed. The K562 cell line was used as a positive control for SET overexpression and hyperphosphorylation of Y-307 of PP2A. N1-N5 indicates normal controls; R, patient sample at relapse; PT, patient sample after treatment; D, patient sample at diagnosis; and C+, K562 cells as positive control.

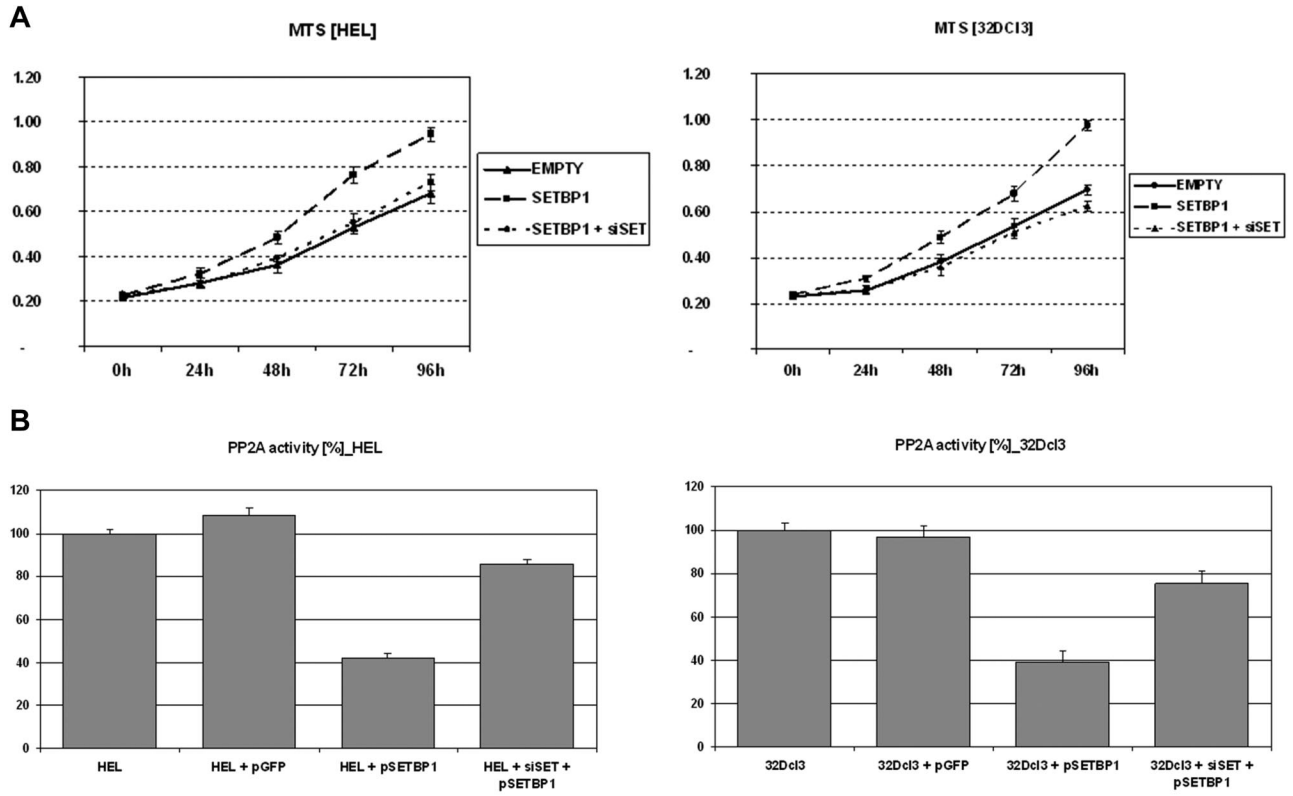


Figure 4. Effect of SETBP1 on cell proliferation and PP2A activity. (A) MTS assays showing the effect of ectopic SETBP1 expression alone or with SET-specific siRNAs on the growth of HEL and 32Dcl3. (B) PP2A phosphatase assay in HEL and 32Dcl3 cells either not transfected or expressing the empty vector as a control, SETBP1 alone, or SETBP1 with SET down-regulated by *SET*-specific siRNAs.

induction therapy. High-dose cytarabine, and autologous or allogenic stem cell transplantation when possible, were used as consolidation therapy. All patients provided informed consent. *SETBP1* was overexpressed in 53 of 192 patients (27.6%). The prevalence in cytogenetic prognostic groups was as follows: good, 24% (7 of 29); intermediate, 18.5% (17 of 92); and poor, 40.8% (29 of 71). We found genetic aberrations associated with *SETBP1* overexpression: monosomy 7 ($P = .007$), chromosome 18 aberrations ($P = .031$), and *EVI1* overexpression ($P = .001$). An inverse correlation was observed between *SETBP1* overexpression and normal karyotype ($P = .010$), and *NPM1* mutations in patients with wild-type *FLT3* ($P = .045$; Table 2).

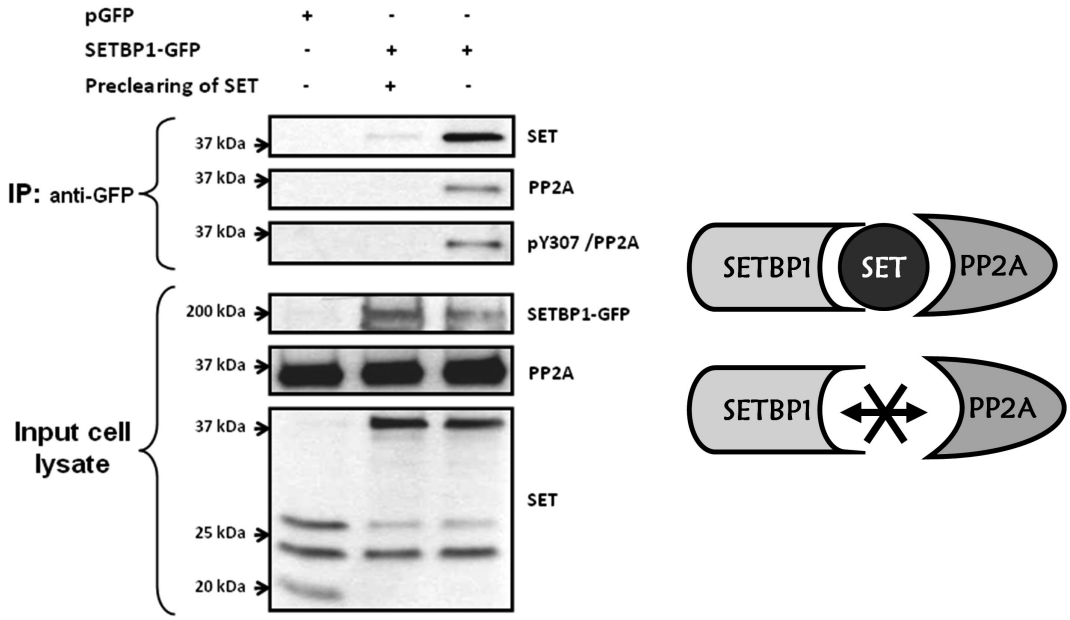


Figure 5. PP2A interacts with SET-SETBP1 heterodimer but not directly with SETBP1. Cell extracts expressing SETBP1-GFP (lanes 2 and 3) were immunoprecipitated with anti-GFP followed by Western blotting with anti-SET and anti-PP2A. Cell extract without SETBP1-GFP expression was used as negative control (lane 1). One of the cell extracts expressing SETBP1-GFP was precleared of SET using anti-SET antibodies bound to Sepharose-protein G before the immunoprecipitation (lane 2).

Table 1. Clinical and molecular characteristics of a series of 192 patients with AML

Characteristic	No. (%)
Sex	
Male	99 (53)
Female	88 (47)
No data	5
Age	
Less than 60 years	92 (50.3)
More than 60 years	91 (49.7)
No data	9
Complete remission	
No	47 (36.7)
Yes	81 (63.3)
No data	64
Diagnosis	
AML-M0	21 (10.9)
AML-M1	35 (18.2)
AML-M2	38 (19.8)
AML-M3	7 (3.6)
AML-M4	33 (17.2)
AML-M5	36 (18.8)
AML-M6	12 (6.3)
AML-NOS	10 (5.2)
Secondary AML	
No	130 (83.3)
Yes	26 (16.7)
No data	36
Cytogenetic group	
Good	29 (15)
Intermediate	92 (48)
Poor	71 (37)
***SETBP1* overexpression**	
No	139 (72.4)
Yes	53 (27.6)
***WT1* overexpression**	
No	22 (16)
Yes	115 (84)
No data	55
***EVI1* overexpression**	
No	132 (76.7)
Yes	40 (23.3)
No data	20
***NPM1* mutated**	
No	18 (37.5)
Yes	30 (62.5)
No data	144
***FLT3*-ITD**	
No	99 (79.2)
Yes	26 (20.8)
No data	67

AML indicates acute myeloid leukemia.

Prognostic impact of *SETBP1* overexpression in AML

Clinical follow-up data were available for 168 patients (supplemental Table 2), 91 men and 77 women, with a median age of 56 years. Median OS of the global cohort was 31.7 (95% confidence interval, 21.5-41.9 weeks; supplemental Figure 5). As expected, significant differences in OS according to age, cytogenetic group, and complete remission rate were found in this series ($P < .01$). In addition, we found significant differences in OS between patients with and without *SETBP1* overexpression ($P < .01$; supplemental Figure 6). Interestingly, the prognostic impact of *SETBP1* overexpression was particularly evident in patients older than 60 years (Figure 6A). Univariate analysis showed that only complete remission ($P < .01$) and *SETBP1* overexpression ($P = .015$), but not cytogenetic group ($P = .059$), were significant in OS in the group of patients older than 60 years. Multivariate analysis demonstrated that *SETBP1* overexpression was an unfavorable independent factor associated with OS in elderly patients with AML (Table 3).

Only 121 of this series of 168 patients received induction therapy. We also found significant differences in age, cytogenetic group, and complete remission (data not shown) in this series. When we investigated the prognostic impact of *SETBP1* overexpression in this cohort, we found that patients with *SETBP1* overexpression had significantly worse OS ($P = .016$) and EFS ($P = .015$; Figure 6B). We found no differences in DFS ($P = .366$). Sixty-six patients of this series were included in the intermediate cytogenetic risk group, and 47 had normal karyotype. Patients with *SETBP1* overexpression had a worse OS than those without (median, 26 vs 45.9 weeks); however, differences were not statistically significant, probably because of the small number of *SETBP1*$^+$ cases. A worse outcome in DFS (median, 20.9 vs 61.4 weeks) and EFS (median, 23.3 vs 40 weeks) was also observed.

To analyze the molecular events that could lead to *SETBP1* overexpression in the AML cases, we performed a bioinformatic analysis of the proximal promoter of *SETBP1*. We identified several hypothetical binding sites for transcription factors previously implicated in leukemia, such as GATA1, GATA2, EVI1, HMG-1, or AP-1 (supplemental Table 3). Moreover, there is a CpG island within the putative proximal promoter of *SETBP1* that spans 1812 bp and could lead to the hypothetical epigenetic deregulation of *SETBP1* in cases with overexpression of this gene. Interestingly, 6 of the 7 retroviral insertion sites described by Ott et al[20] are located in a region of 200 bp (40513701-40513912), where this CpG island is located (40512982-40514793).

Discussion

We report here a novel mechanism of transformation in acute myeloid leukemia. We first investigated a t(12;18)(p13;q12) involving *ETV6* in a patient with AML. Functionally significant fusions could not be detected. Knowing that *SETBP1* (18q12) was close to the translocation breakpoint, and taking into account that ectopic expression of oncogenes is a mechanism involved in leukemia, we postulated that overexpression of *SETBP1* could be the major event in this case. Indeed, *SETBP1* was overexpressed at diagnosis and in the posttreatment samples of the patient (Figure 1). As activation of *SETBP1* expression by retroviral integration in hematopoietic progenitor cells had been reported to confer a growth advantage leading to clonal expansion[20] and we could establish that *SETBP1* overexpression was a recurrent molecular event with poor prognostic impact in AML, we decided to investigate the leukemogenic mechanism of overexpression of this gene. In this paper, we show that SETBP1 overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein, and leading to the formation of a SETBP1–SET-PP2A complex that results in PP2A inhibition, and therefore promotes the proliferation and expansion of leukemic cells.

SETBP1 encodes a protein of 1542 amino acids and 170 kDa, localized predominantly in the nucleus, as we have confirmed in this study (supplemental Figure 2B). The physiologic function as well as the molecular mechanism by which SETBP1 acts remains unknown. The protein contains a region homologous to the dimerization domain of SKI, and a SET-binding region, although

Table 2. Association between *SETBP1* overexpression and clinical and genetic parameters in 192 patients with AML at diagnosis

	No. of cases	SETBP1$^-$, no. (%)	No. SETBP1$^+$, no. (%)	*P*
SETBP1	192	139 (72.4)	53 (27.6)	
Sex	187	136	51	.511
Male	99	70 (70.7)	29 (29.3)	
Female	88	66 (75)	22 (25)	
Age	183	136	47	.219
Less than 60 years	92	72 (78.3)	20 (21.7)	
More than 60 years	91	64 (70.3)	27 (29.7)	
Complete remission	128	180	50	.196
No	47	33 (70.2)	14 (29.8)	
Yes	81	65 (80.2)	16 (19.8)	
Secondary AML	156	247	56	.354
No	130	101 (77.7)	29 (22.3)	
Yes	26	18 (69.2)	8 (30.8)	
Prognostic group	192	139	53	.006*
Good	29	22 (76)	7 (24)	
Intermediate	92	75 (82.5)	17 (18.5)	
Poor	71	42 (59.2)	29 (40.8)	
Cytogenetic group	192	139	53	
Normal karyotype				.010*
Yes	63	53 (84)	10 (16)	
No	128	85 (66.4)	43 (33.6)	
Trisomy 8				.943
Yes	18	13 (72.2)	5 (27.8)	
No	163	119 (73)	44 (27)	
Chromosome 18 aberrations				.031*
Yes	11	5 (45.4)	6 (54.6)	
No	169	127 (75)	42 (25)	
Monosomy 7				.007*
Yes	27	14 (51.9)	13 (48.1)	
No	155	119 (76.8)	36 (23.2)	
der(7q)				.318
Yes	16	10 (62.5)	6 (37.5)	
No	166	123 (74.1)	43 (25.9)	
***WT1* overexpression**	137	108	29	.345
No	22	19 (86.7)	3 (13.6)	
Yes	115	89 (77.4)	26 (22.6)	
***EVI1* overexpression**	172	126	46	.001*
No	132	108 (81.8)	24 (18.2)	
Yes	40	18 (45)	22 (55)	
***MDS1EVI1* overexpression**	152	109	43	.012*
No	138	103 (74.6)	35 (25.4)	
Yes	14	6 (42.9)	8 (57.1)	
***NPM1* mutated and FLT3 wt**	32	28	4	.014*
No	12	8 (66.7)	4 (33.3)	
Yes	20	20 (100)	0 (0.0)	
***FLT3*-ITD**	125	100	25	.760
No	99	78 (78.8)	21 (21.2)	
Yes	26	22 (84.6)	4 (15.4)	

AML indicates acute myeloid leukemia.
*Significant values.

the functional significance of these interactions has not been determined.[21] The protein SET (I2PP2A/TAF-Iβ) inhibits PP2A through the phosphorylation of the PP2Ac tyrosine-307.[24] Indeed, SET is overexpressed in multiple solid tumors[25] and in chronic myeloid leukemia where its correlation has been demonstrated with the expression and oncogenic activity of BCR-ABL, leading to PP2A inhibition.[23]

Here, we demonstrate that SETBP1 overexpression increased the 39-kDa full-length SET, probably because the SETBP1-SET interaction protects SET from protease-mediated cleavage. As a consequence, we detected a decrease in the 27-, 24-, and 20-kDa SET forms (Figure 2A). It has been reported that SET inhibits the DNase activity of the tumor suppressor NM23-H1 and that the cleavage of SET by Granzyme A during the cytotoxic T lymphocyte-induced apoptosis releases NM23-H1 from inhibition and triggers NM23-H1 to translocate into the nucleus, where it nicks the DNA.[26,27] Interestingly, the cleavage of SET by Granzyme A generates 3 polypeptides with similar molecular weight to those we detected. Although more studies are needed to clarify this point, our results suggest a possible role for SETBP1 impairing the cleavage of SET by Granzyme A in the apoptosis caspase-independent pathway induced by cytotoxic T lymphocytes. This could point to a novel defense mechanism in leukemic cells.

PP2A is a major protein phosphatase implicated in many cell processes,[28-32] and its loss of function has been associated with cell

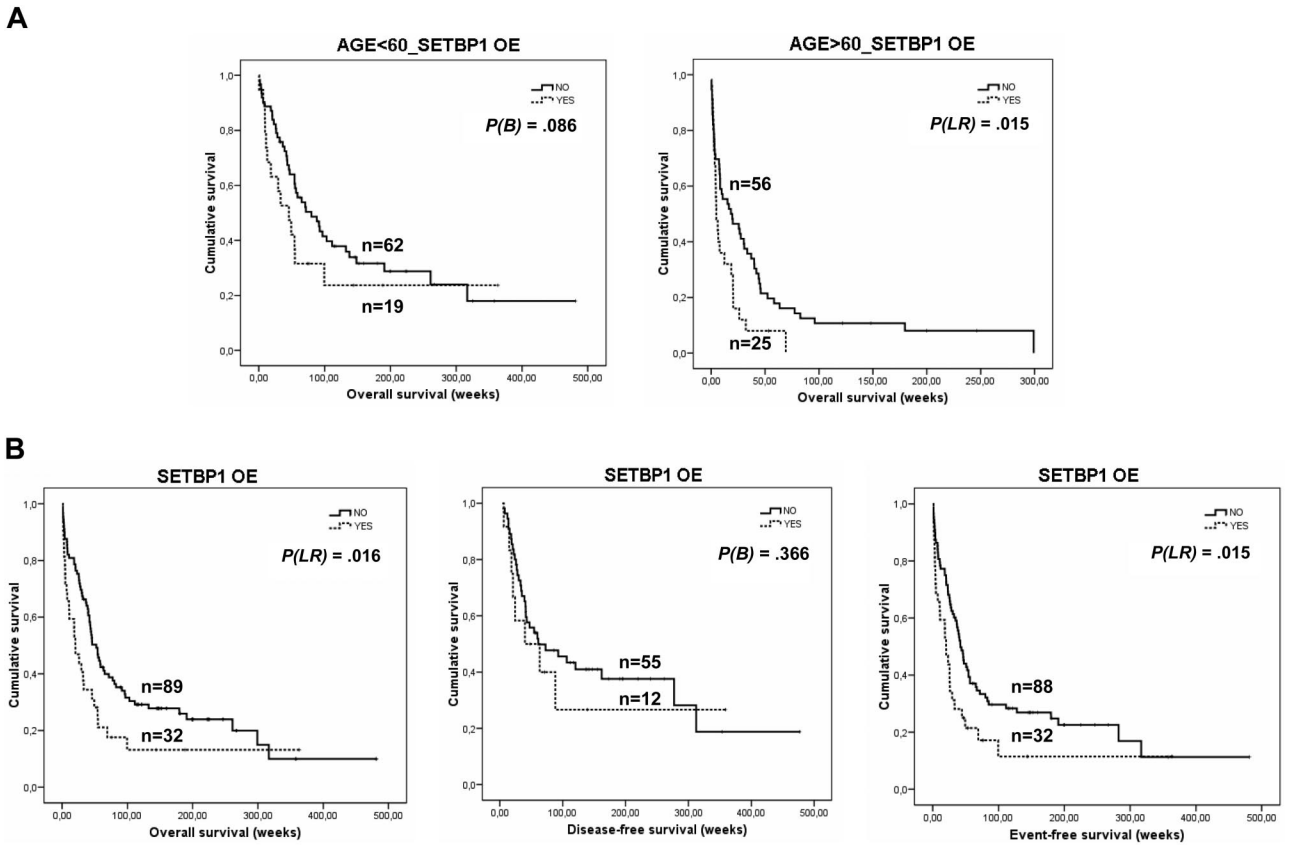


Figure 6. *SETBP1* overexpression and cumulative survival in AML. (A) Kaplan-Meier analyses of OS for *SETBP1* in the series of age-stratified 168 patients with AML, and clinical follow-up data available. Patients older than 60 years with *SETBP1* overexpression showed a poorer outcome compared with patients with no *SETBP1* overexpression. (B) Kaplan-Meier analyses of OS, DFS, and EFS for *SETBP1* overexpression in a series of 121 patients with AML and clinical follow-up data available who received induction therapy (note that this group of 121 patients is included in the previous series of 168 patients in panel A).

transformation.[33,34] Because SET is a potent inhibitor of PP2A and SETBP1 overexpression alters the level of SET, we analyzed the effect of SETBP1 overexpression on PP2A. In addition to the previously reported SETBP1-SET and SET-PP2A complexes, we demonstrated the formation of a SETBP1–SET-PP2A heterotrimeric complex, in which PP2A is phosphorylated and, therefore, inhibited (Figure 5). Our results suggest that this is the molecular mechanism by which PP2A is inhibited in patients with *SETBP1* overexpression and AML because PP2A and SETBP1 did not interact in the absence of SET (Figure 5). Interestingly, we only detected the full-length SET form in the immunoprecipitate, indicating that the shorter processed forms are not present in the SETBP1–SET-PP2A complex. It has been reported that these forms conserve their inhibitory activity of PP2A, but it remains unclear whether this activity is similar to that of full-length SET[35]; our results suggest that the processed forms may have lower PP2A inhibitory activity and that SETBP1 may increase the inhibition of PP2A by protecting the full-length SET protein, and permitting its interaction with PP2A.

Moreover, SET is a key modulator of DNA replication, chromatin remodeling and gene transcription,[36,37] differentiation,[38] and cell-cycle regulation.[39] We postulate that the protecting role that SETBP1 overexpression plays on SET might reflect changes in the expression patterns of genes whose acetylation depends on the presence of the full-length SET protein because 39 kDa SET, but not the processed forms, has been reported to inhibit histone acetylation and to bind to HuR, which stabilizes immediate early gene mRNAs.[25,36,40] Our results showed that the SETBD region is critical for this function (Figure 2B); therefore, the interaction of SETBP1 and SET could have other effects apart from the inhibition of PP2A.

Table 3. Multivariate analysis of clinical and biologic variables in the group of patients with AML older than 60 years

Variable	Subset	Hazard ratio (95% confidence interval)	*P*
Complete remission	No/yes	1/0.218 (0.110-0.430)	.001
SETBP1 overexpression	No/yes	1/2.311 (1.173-4.553)	.015

AML indicates acute myeloid leukemia.

To assess the prevalence and the prognostic significance of *SETBP1* overexpression, we analyzed the expression of this gene in 192 patients with AML at diagnosis, and we found that *SETBP1* overexpression was a recurrent event in AML, accounting for 27.6% of all cases. Our results show that *SETBP1* overexpression predicts shorter OS and that the impact on prognosis is especially significant in patients older than 60 years (Figure 6A). AML is a disease of the elderly, with a median age at diagnosis more than 60 years. However, the gradual improvements achieved in the last 2 decades have been mainly focused on the group of patients younger than 60 years, whereas there has been no change in the OS of patients older than 60 years, probably because of both patient- and disease-specific factors, although this subgroup represents two-thirds of the total number of cases.[41] It is therefore important to identify genetic markers that could predict prognosis in this subgroup of patients, as well as to advance our knowledge of disease biology to develop novel targeted therapies. Our data suggest that *SETBP1* overexpression could distinguish 2 subgroups of AML elderly patients; furthermore, it could be a predictive factor

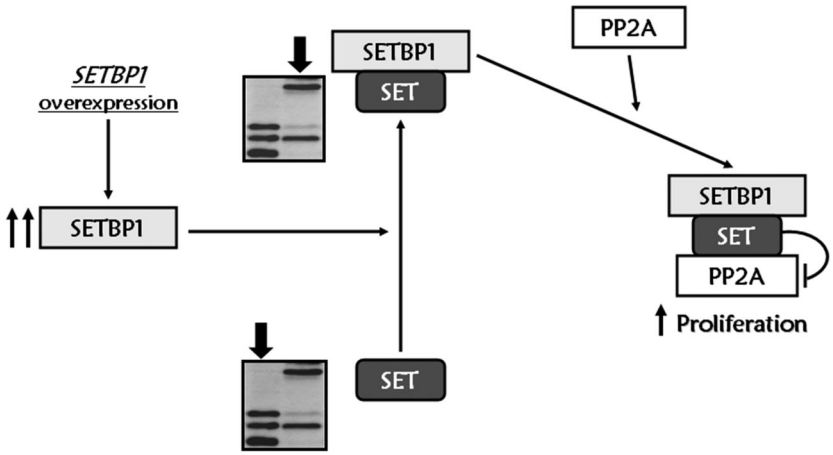


Figure 7. Proposed molecular model for SETBP1 signaling pathway. SETBP1 overexpression protects SET from protease cleavage, increasing the amount of full-length SET protein, and leading to the formation of a SETBP1–SET-PP2A complex that results in PP2A inhibition and therefore promotes the proliferation of leukemic cells.

of response to PP2A activators, such as FTY720, which has been proposed as a future alternative for treating blast-crisis chronic myeloid leukemia and Philadelphia-positive acute lymphocytic leukemia.[42]

Although multivariate analysis confirmed the negative prognostic impact of *SETBP1* overexpression in our series, it was associated with other poor prognostic markers, such as monosomy 7 and *EVI1* overexpression. AML is a multistep genetic disease, and the activation of *SETBP1* may cooperate with additional mutations to induce leukemia.[20] In the patient with t(12;18), we found no *FLT3*, *NPM1*, or *JAK2* mutations, although trisomy 19 was observed in the blast cells of the patient as a secondary event that would cooperate in the progression of the disease. Moreover, posttreatment samples showed overexpression of *SET* and *ETV6*, which could act synergistically with *SETBP1* overexpression (supplemental Figure 3). The mechanisms by which *SETBP1* is overexpressed in the series analyzed remain unknown. However, bioinformatic analysis of the putative promoter region of *SETBP1* allowed us to identify several transcription factors previously implicated in leukemia (supplemental Table 3); so we postulate that their deregulation could promote *SETBP1* overexpression. FISH analysis suggests that the breakpoint in the patient with the t(12;18) is located upstream to the transcription start site of *SETBP1*, close to the retroviral insertion sites described by Ott et al.[20] Moreover, 6 of the 7 retroviral insertion sites described in that paper are located in a region of 200 bp (40513701-40513912), where we have found a CpG island (40512982-40514793). Taken together, this suggests that this is an important region for the transcriptional regulation of the *SETBP1* gene and that epigenetic aberrations could be another mechanism of *SETBP1* overexpression in patients with AML without 18q aberrations. Further studies to confirm this and to analyze the promoter region of *SETBP1* are in progress.

In conclusion, we report a novel mechanism of leukemic transformation. Overexpression of SETBP1 promotes an increase in full-length SET levels, impairing the phosphatase activity of the tumor suppressor PP2A through the formation of a SETBP1–SET-PP2A complex, and promoting the proliferation of cells (Figure 7). Besides, we demonstrate that SETBP1 overexpression protects SET from protease cleavage, which could have important effects on both the histone acetylation inhibitory activity of SET and Granzyme A–mediated caspase-independent apoptosis induced by cytotoxic T lymphocytes. Furthermore, deregulation of *SETBP1* by translocations or other unknown mechanisms seems to play a crucial role in the leukemic transformation of AML. We have shown that *SETBP1* overexpression is a recurrent molecular event with independent prognostic value in AML, especially in the subgroup of elderly patients. Further research into the physiologic function of this gene will contribute to a better understanding of the multiple steps that give rise to AML.

Acknowledgments

The authors thank Elizabeth Guruceaga, of the Unit of Proteomics, Genomics and Bioinformatics of Center for Applied Medical Research, for the bioinformatics analysis, and Enrique Andreu for useful discussion.

This work was supported by the Ministerio de Educación y Ciencia, Ministerio de Ciencia e Innovación (PI081687, M.D.O.; and SAF2007-61827, C.B.), Departamento de Salud del Gobierno de Navarra (14/2008), ISCIII-RTICC (RD06/0020/0078), and Fundación para la Investigación Médica Aplicada y UTE (Spain).

Authorship

Contribution: I.C., F.J.B., L.G.-O., and N.M. performed research; C.V. performed FISH analyses; F.J.N. and E.B. contributed to bioinformatics and statistical analyses; J.R. and M.J.C. made available clinical histories and patient samples; and I.C., C.B., and M.D.O. designed research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: María D. Odero, Division of Oncology, Center for Applied Medical Research, University of Navarra, Pio XII-55 31008-Pamplona, Spain; e-mail: modero@unav.es.

References

1. Dohner H. Implication of the molecular characterization of acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program.* 2007:412-419.
2. Mrózek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev.* 2004;18(2):115-136.
3. Loriaux MM, Levine RL, Tyner JW, et al. High-throughput sequence analysis of the tyrosine kinome in acute myeloid leukemia. *Blood.* 2008;111(9):4788-4796.
4. Tomasson MH, Xiang Z, Walgren R, et al. Somatic mutations and germline sequence variants

in the expressed tyrosine kinase genes of patients with de novo acute myeloid leukemia. *Blood.* 2008;111(9):4797-4808.

5. Schoch C, Kohlmann A, Schnittger S, et al. Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. *Proc Natl Acad Sci U S A.* 2002;99(15):10008-10013.
6. Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med.* 2004;350(16):1605-1616.
7. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med.* 2004;350(16): 1617-1628.
8. Raghavan M, Lillington DM, Skoulakis S, et al. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. *Cancer Res.* 2005;65(2):375-378.
9. Paulsson K, Heidenblad M, Strombeck B, et al. High-resolution genome-wide array-based comparative genome hybridization reveals cryptic chromosome changes in AML and MDS cases with trisomy 8 as the sole cytogenetic aberration. *Leukemia.* 2006;20(5):840-846.
10. Rücker FG, Bullinger L, Schwaenen C, et al. Disclosure of candidate genes in acute myeloid leukemia with complex karyotypes using microarray-based molecular characterization. *J Clin Oncol.* 2006;24(24):3887-3894.
11. Suela J, Alvarez S, Cifuentes F, et al. DNA profiling analysis of 100 consecutive de novo acute myeloid leukemia cases reveals patterns of genomic instability that affect all cytogenetic risk groups. *Leukemia.* 2007;21(6):1224-1231.
12. Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature.* 2008;456(7218):66-72.
13. Cools J, Mentens N, Odero MD, et al. Evidence for position effects as a variant ETV6-mediated leukemogenic mechanism in myeloid leukemias with a t(4;12)(q11-q12;p13) or t(5;12)(q31;p13). *Blood.* 2002;99(5):1776-1784.
14. Odero MD, Vizmanos JL, Roman JP, et al. A novel gene, MDS2, is fused to ETV6/TEL in a t(1;12)(p36.1;p13) in a patient with myelodysplastic syndrome. *Genes Chromosomes Cancer.* 2002;35(1):11-19.
15. Nucifora G, Laricchia-Robbio L, Senyuk V. EVI1 and hematopoietic disorders: history and perspectives. *Gene.* 2006;368:1-11.
16. Gallay N, Dos Santos C, Cuzin L, et al. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia.* 2009;23(6):1029-1038.
17. Coessens B, Thijs G, Aerts S, et al. INCLUSive: a web portal and service registry for microarray and regulatory sequence analysis. *Nucleic Acids Res.* 2003;31(13):3468-3470.
18. Bryne JC, Valen E, Tang MH, et al. JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic Acids Res.* 2008;36(Database issue):D102-D106.
19. Matys V, Kel-Margoulis OV, Fricke E, et al. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res.* 2006;34(Database issue):D108-D110.
20. Ott MG, Schmidt M, Schwarzwaelder K, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med.* 2006;12(4):401-409.
21. Minakuchi M, Kakazu N, Gorrin-Rivas MJ, et al. Identification and characterization of SEB, a novel protein that binds to the acute undifferentiated leukemia-associated protein SET. *Eur J Biochem.* 2001;268(5):1340-1351.
22. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science.* 1992;257(5074): 1261-1264.
23. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell.* 2005;8(5):355-368.
24. Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem.* 1996; 271(19):11059-11062.
25. Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. *J Biol Chem.* 2002; 277(28):25026-25031.
26. Beresford PJ, Zhang D, Oh DY, et al. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem.* 2001;276(46): 43285-43293.
27. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell.* 2003;112(5):659-672.
28. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J.* 2001;353(3):417-439.
29. Wang GL, Iakova P, Wilde M, Awad S, Timchenko NA. Liver tumors escape negative control of proliferation via PI3K/Akt-mediated block of C/EBP alpha growth inhibitory activity. *Genes Dev.* 2004; 18(8):912-925.
30. Kawabe T, Muslin AJ, Korsmeyer SJ. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G_2/M cell-cycle checkpoint. *Nature.* 1997;385(6615):454-458.
31. Mumby M. PP2A: unveiling a reluctant tumor suppressor. *Cell.* 2007;130(1):21-24.
32. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med.* 2008;14(4):152-160.
33. Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. *Curr Opin Genet Dev.* 2005;15(1):34-41.
34. Schönthal AH. Role of serine/threonine protein phosphatase 2A in cancer. *Cancer Lett.* 2001; 170(1):1-13.
35. Beresford PJ, Xia Z, Greenberg AH, Lieberman J. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity.* 1999;10(5):585-594.
36. Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell.* 2001;104(1):119-130.
37. Kutney SN, Hong R, Macfarlan T, Chakravarti D. A signaling role of histone-binding proteins and INHAT subunits pp32 and Set/TAF-Ibeta in integrating chromatin hypoacetylation and transcriptional repression. *J Biol Chem.* 2004;279(29): 30850-30855.
38. Kandilci A, Mientjes E, Grosveld G. Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937. *Leukemia.* 2004;18(2):337-340.
39. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, et al. The SET protein regulates G_2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. *J Biol Chem.* 2003;278(2):1158-1164.
40. Brennan CM, Gallouzi IE, Steitz JA. Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J Cell Biol.* 2000;151(1):1-14.
41. Büchner T, Berdel WE, Haferlach C, et al. Age-related risk profile and chemotherapy dose response in acute myeloid leukemia: a study by the German Acute Myeloid Leukemia Cooperative Group. *J Clin Oncol.* 2009;27(1):61-69.
42. Neviani P, Santhanam R, Oaks JJ, et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest.* 2007;117(9):2408-2421.

Figure S1. Genetic characterization of a patient with AML-M5 and a t(12;18)(p13;q12) - (A) Partial karyotype of the patient. Derivative chromosomes are indicated by arrows; (B) FISH analysis of the patient showing that ETV6 was rearranged.

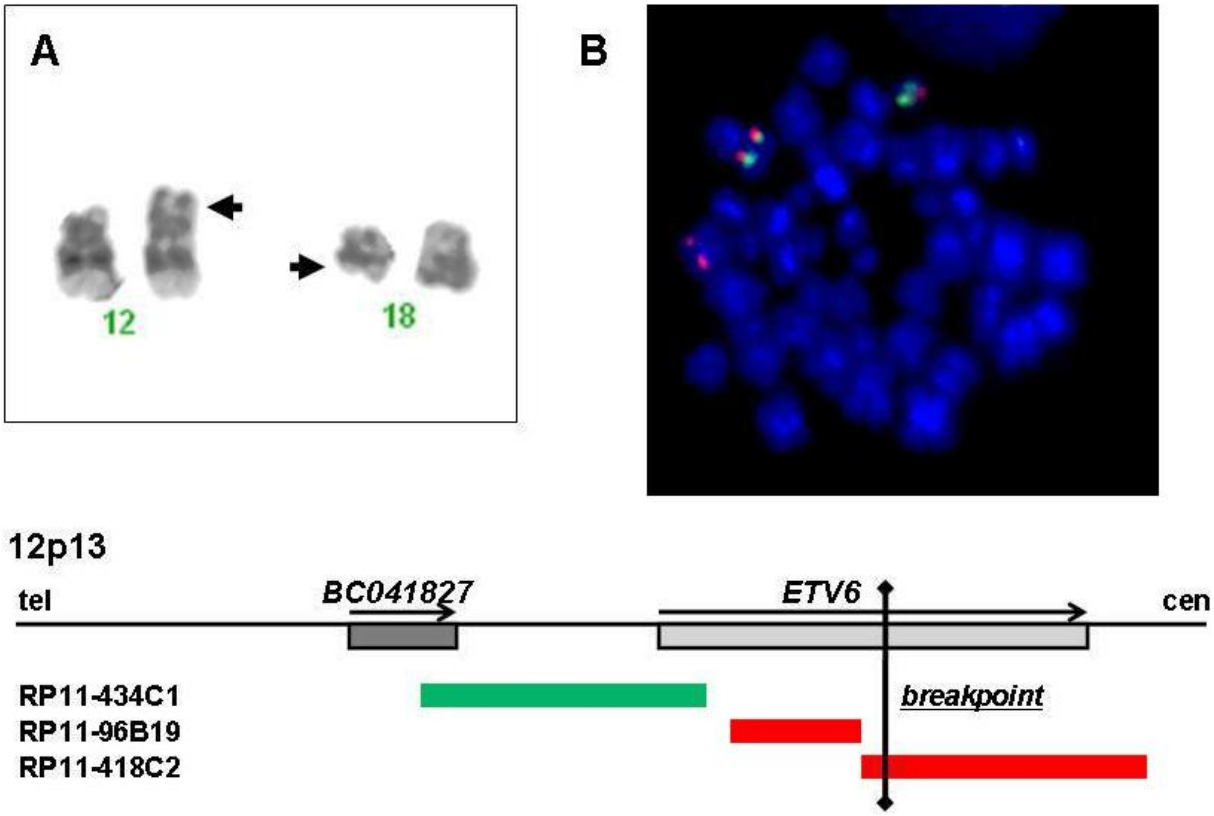


Figure S2 - (A) Quantification of SETBP1 expression levels by QRT-PCR (ΔΔCt method) in parental HEK293 cells and HEK293 cells expressing SETBP1 or the empty vector. (B) SETBP1 shows mainly nuclear intracellular location. (C) HEK293 cells were transiently transfected with SET-GFP and harvested at different times. SET-GFP expression began 9 hours after transfection, with two shorter forms were appearing progressively.

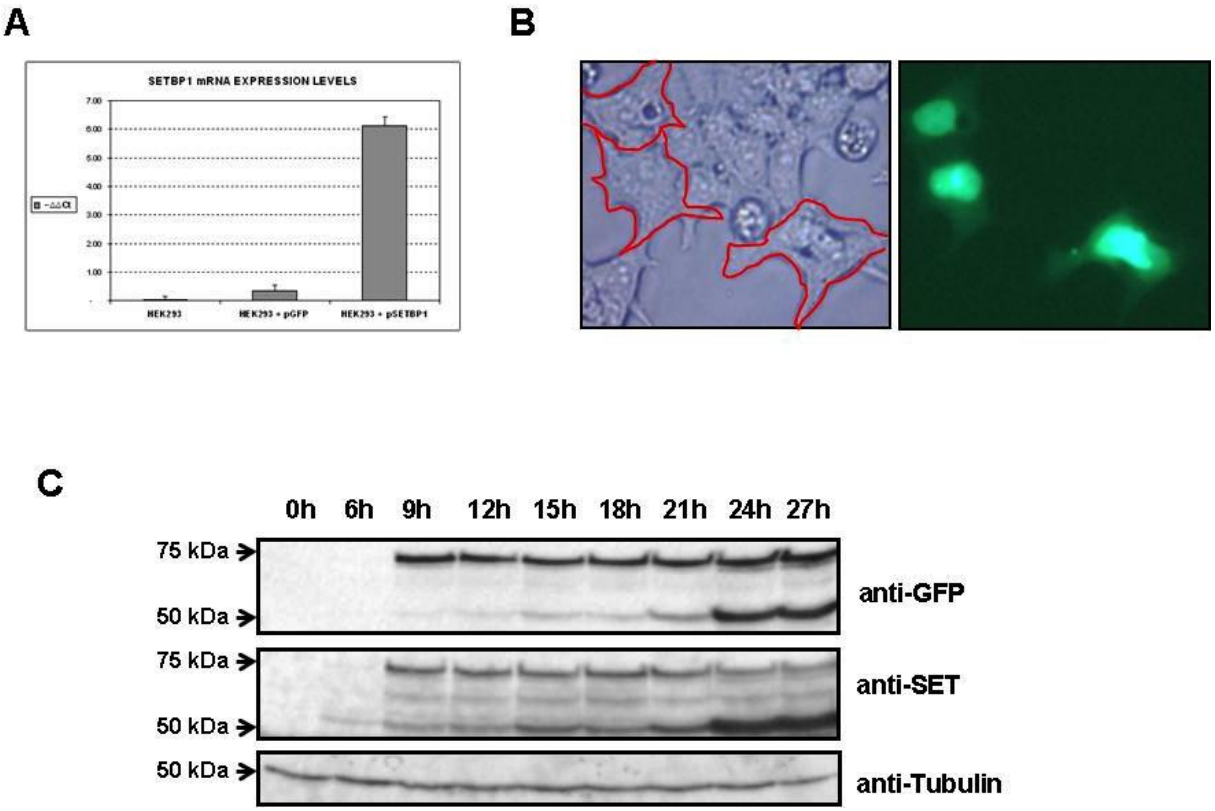


Figure S3. Quantification of SET and ETV6 expression levels by QRT-PCR (ΔΔCt method) in the patient samples at diagnosis, post-treatment control and relapse

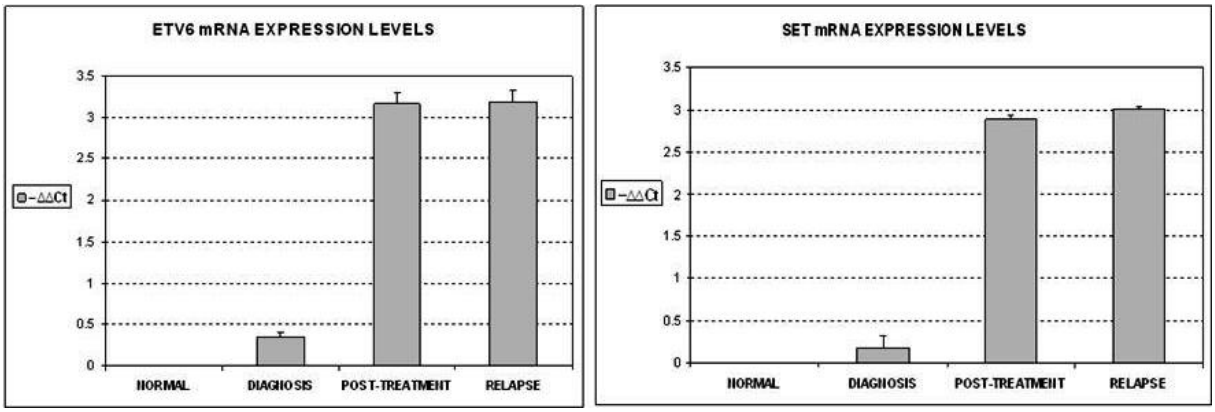


Figure S4 (A) Western blot analysis showing the levels of immunoprecipitated PP2A from the cell lines used in the phosphatase assays showed in Fig. 2E and 4B; (B) Western blot analysis and relative SET expression by QRT-PCR of HEL and 32Dcl3 cells transfected with a control siRNA or siSET.

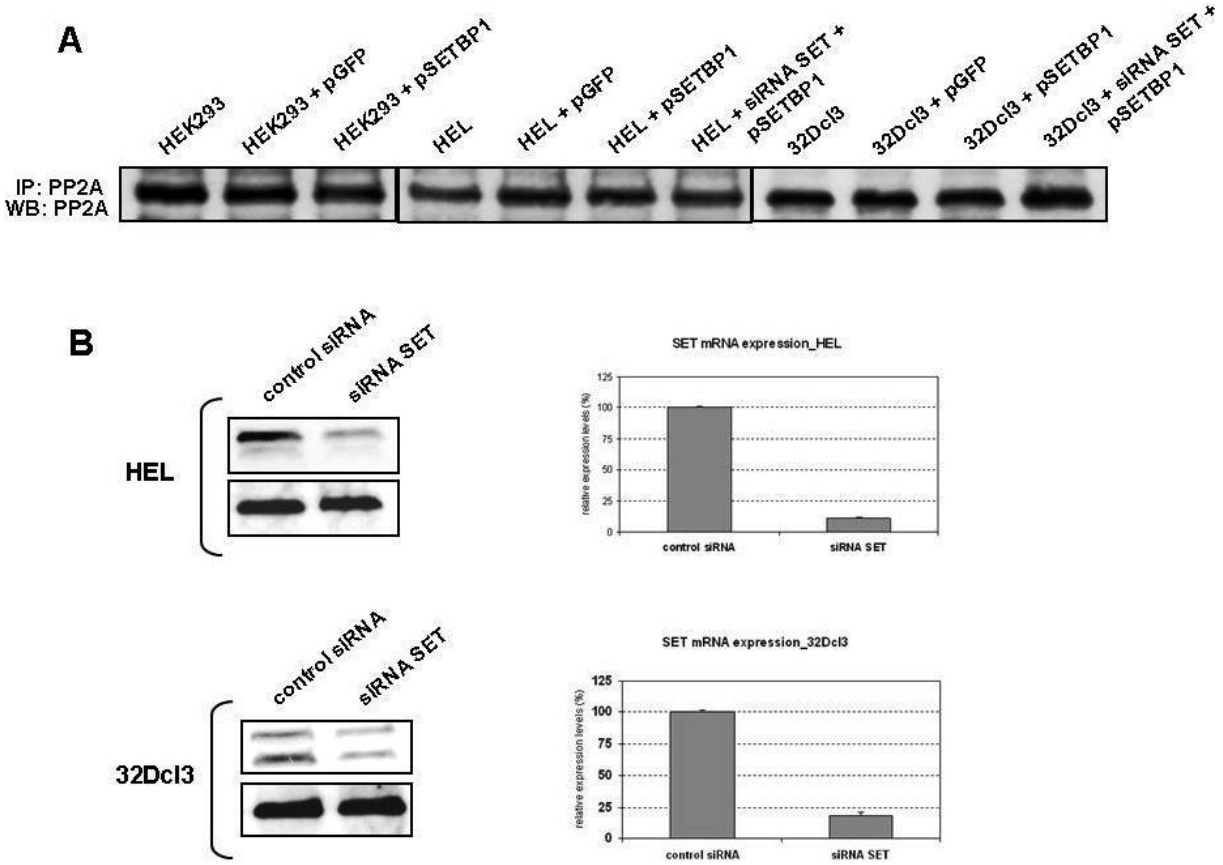
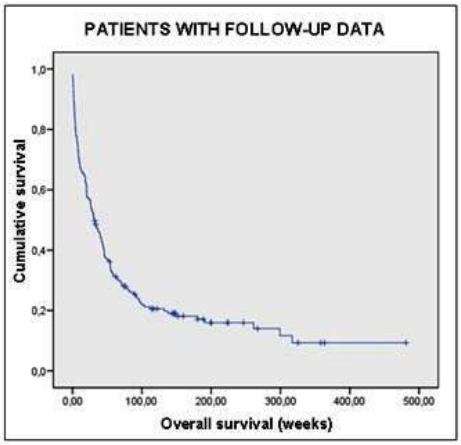


Figure S5 (A) Survival graph of the 168 AML patients with follow-up data available; (B) Box-plots showing SETBP1 expression levels in the groups of AML patients without (n=139), and with SETBP1 overexpression (n=53).

A



Time (weeks)	Cumulative Proportion Surviving at the time		Nº of Cumulative Events	Cases at risk
	Estimate	Std. Error		
26	55.1%	3.8%	76	92
52	36.8%	3.8%	108	60
104	21.2%	3.3%	137	31
156	11.4%	3.1%	149	19
206	6.6%	3.2%	157	11
260	4.8%	3.2%	160	8

B

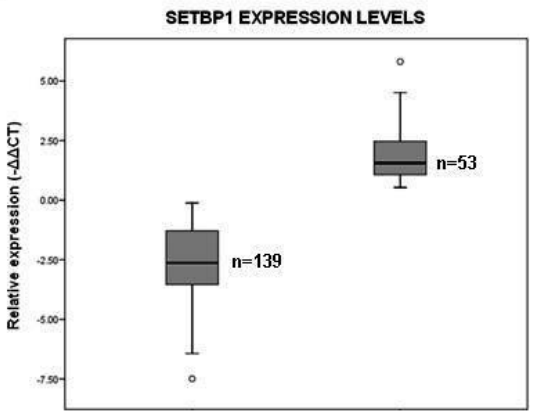
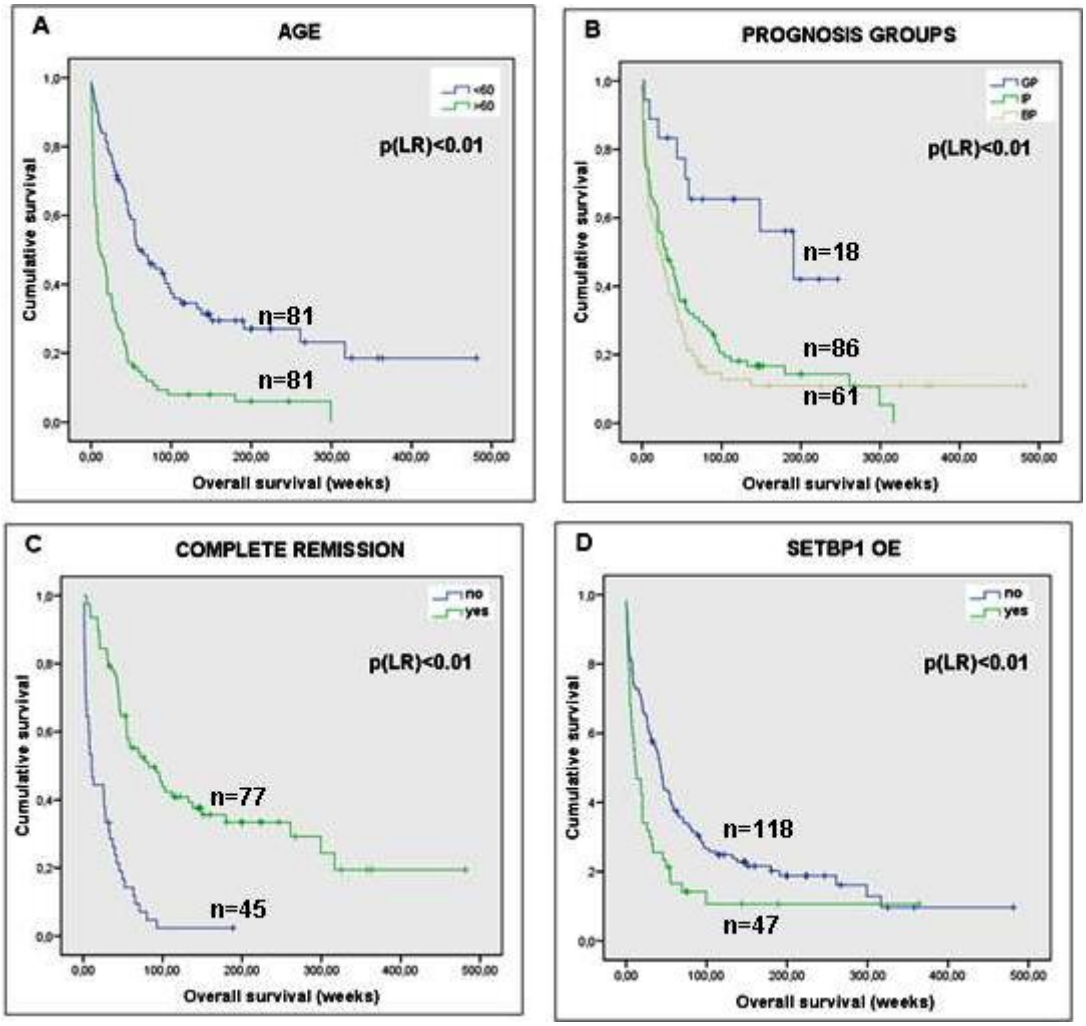


Figure S6. Kaplan-Meier analyses of overall survival in the cohort of 168 patients with clinical follow-up data available show an inferior outcome (A) patients older than 60 years; (B) patients included in the intermediate and poor prognosis groups; (C) patients who did not achieve CR; (D) and patients with SETBP1 overexpression



Leukemia (2011), 1–9

www.nature.com/leu

ORIGINAL ARTICLE

PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect

I Cristóbal[1,2], L Garcia-Orti[1], C Cirauqui[1], MM Alonso[1], MJ Calasanz[2] and MD Odero[1,2]

[1]Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Navarra, Spain and [2]Department of Genetics, University of Navarra, Pamplona, Navarra, Spain

Protein phosphatase 2A (PP2A) is a human tumor suppressor that inhibits cellular transformation by regulating the activity of several signaling proteins critical for malignant cell behavior. PP2A has been described as a potential therapeutic target in chronic myeloid leukemia, Philadelphia chromosome-positive acute lymphoblastic leukemia and B-cell chronic lymphocytic leukemia. Here, we show that PP2A inactivation is a recurrent event in acute myeloid leukemia (AML), and that restoration of PP2A phosphatase activity by treatment with forskolin in AML cells blocks proliferation, induces caspase-dependent apoptosis and affects AKT and ERK1/2 activity. Moreover, treatment with forskolin had an additive effect with Idarubicin and Ara-c, drugs used in standard induction therapy in AML patients. Analysis at protein level of the PP2A activation status in a series of patients with AML at diagnosis showed PP2A hyperphosphorylation in 78% of cases (29/37). In addition, we found that either deregulated expression of the endogenous PP2A inhibitors SET or CIP2A, overexpression of SETBP1, or downregulation of some PP2A subunits, might be contributing to PP2A inhibition in AML. In conclusion, our results show that PP2A inhibition is a common event in AML cells and that PP2A activators, such as forskolin or FTY720, could represent potential novel therapeutic targets in AML.
Leukemia advance online publication, 14 January 2011; doi:10.1038/leu.2010.294
Keywords: AML; PP2A; forskolin; therapy; SET

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disease that disrupts normal hematopoiesis. Leukemic cells are characterized by a block in differentiation and apoptosis, together with an enhanced proliferation. Despite progressive advances in our understanding of the molecular biology of AML, patient outcomes are still very poor. Complete remission occurs in up to half of these patients; however, relapse is generally expected and prognosis is dismal.[1] Therefore, it is necessary to develop more effective treatment strategies to improve the survival of these patients.[2]

The unrestricted growth of transformed cells is caused by the cumulative deregulation of multiple cellular pathways involved in normal growth control.[3] The ubiquitously expressed protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase that accounts for most of the serine/threonine phosphatase activity in eukaryotic cells, and participates in many mammalian signaling pathways.[4] PP2A represents a family of heterotrimeric holoenzyme complexes consisting of an active core composed of the scaffold PP2A-A subunit, the catalytic PP2A-C subunit and a regulatory PP2A-B subunit. There are two closely related isoforms of the PP2A-A (Aα/PPP2R1A and Aβ/PPP2R1B),[5,6] and of the PP2A-C (Cα/PPP2CA and Cβ/PPP2CB) subunits.[7,8] The scaffold subunit mediates interaction of the core dimer with a wide variety of regulatory B subunits that regulate both the specific substrate and the localization of the holoenzyme. Four unrelated families of regulatory B subunits have been identified, including at least 26 different alternative transcripts and splice forms.[9] Therefore, PP2A has the ability to form complexes with many different substrates.[3,10] A variety of mechanisms that inhibit PP2A are present in transformed cells, including alterations in structural or regulatory PP2A subunits, and also the overexpression of specific endogenous inhibitors.[3,10] Somatic mutations of the PP2A structural subunits Aα and Aβ have been described in several types of cancer, causing a defective binding of the B and C subunits and thus inhibiting PP2A activity.[11–20] Moreover, suppression of PP2A Aβ expression permits immortalized human cells to achieve a tumorigenic state through the deregulation of RalA GTPase activity. Cancer-associated Aβ mutants fail to reverse this tumorigenic phenotype, indicating that these mutants function as null alleles.[21] In addition, both Aα mutants and Aα downregulation lead to a functional haploinsufficiency that seems to induce human cell transformation by activating AKT/PI3 K signaling pathway.[22,23] However, it is likely that different sets of genetic aberrations during tumor formation require the loss of different PP2A holoenzyme complexes for the tumor progression, and this would involve the regulatory subunits that are having a key role directing PP2A to dephosphorylate and regulate key tumor suppressors or oncogenes.[9] In this regard, several members of the B56 family of regulatory PP2A subunits seems to have a main role in directing PP2A potential tumor-suppressive activity.[24–30]

With regard to the endogenous PP2A inhibitors, upregulation of SET by the BCR/ABL oncogene leads to the suppression of PP2A, and contributes to leukemogenesis in chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia.[31,32] In addition, Junttila *et al.*[33] provide strong evidence that cancerous inhibitor of PP2A (CIP2A) selectively targets PP2A associated with c-Myc to inhibit its phosphatase activity and protect Ser62 from dephosphorylation. Interestingly, CIP2A expression is upregulated in transformed cell lines and cancer tissue samples. Finally, it has been reported that JAK2 directly phosphorylate PP2A at tyrosine 307 of its catalytic subunit, making PP2A inactive.[34]

Few studies have investigated the role of PP2A in AML. Gallay *et al.*[35] reported that the intensity of phospho-Akt on Thr308 in AML was significantly correlated with high-risk cytogenetics,

Correspondence: Dr MD Odero, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, C/ Pio XII-55, Pamplona, Navarra 31008, Spain.
E-mail: modero@unav.es
Received 22 June 2010; revised 27 October 2010; accepted 15 November 2010

particularly with a complex karyotype, and they found correlation between decreased PP2A activity and Thr308 phosphorylation in this subgroup (seven cases). Moreover, a recent study shows that activating c-KIT mutations inhibit PP2A, and that reactivation of PP2A effectively suppresses the *in vitro* and *in vivo* growth of imatinib-sensitive and imatinib-resistant c-KIT-positive cells, indicating that functional inactivation of PP2A tumor suppressor activity could represent a key step in the induction and maintenance of KIT-positive leukemias.[36] Our group has previously reported that SETBP1 overexpression is a recurrent event in AML, which impairs PP2A activity via SET and promotes proliferation of AML cells.[37] In addition, it has been reported that the activity of PP1 and PP2A is enhanced in the arsenic sulphide-induced differentiation of the AML cell line HL-60,[38] and that the alkylphosphocholine erucylphosphohomocholine is cytotoxic to AML cells through JNK- and PP2A-dependent mechanisms.[39] Interestingly, PP2A activators such as FTY720 in CML, acute lymphoblastic leukemia and chronic lymphocytic leukemia, and forskolin in CML show promising anti-leukemic effects in both *in vitro* and *in vivo* models.

In this study, we show that PP2A activity is reduced in both myeloid Philadelphia-negative cell lines and AML patient samples. Treatment with the PP2A-activator forskolin restores PP2A activity, affecting proliferation and inducing changes in the phosphorylation status of AKT and ERK1/2. Moreover, we found an additive effect between PP2A activation by forskolin and the chemotherapy reagents Idarubicin and cytosine arabinoside (Ara-c), suggesting that treatment with PP2A activators could be a therapeutic target in AML in combination with standard induction therapy. Finally, deregulated expression of endogenous PP2A inhibitors, together with aberrations affecting the expression of PP2A subunits, were identified as possible mechanisms of PP2A inhibition in AML.

Materials and methods

Cell cultures

EOL-1, HL-60, Kasumi-1, OCI-AML2, MOLM13, MV4-11, HEL, KG-1, KYO-1, K562 and MEG-01 cells were maintained in RPMI-1640 (Invitrogen, Breda, The Netherlands) with 10% fetal bovine serum (FBS); NOMO-1 and KU-812 in RPMI-1640 with 20% FBS; F-36P in RPMI-1640 with 20% FBS, and 10 ng/ml GM-CSF; UT-7 in alpha-MEM (Invitrogen) with 20% FBS and 5 ng/ml GM-CSF; MUTZ-3 in 80% alpha-MEM with 20% FBS and 10 ng/ml GM-CSF; and TF-1 in RPMI-1640 with 20% FBS and 10 ng/ml GM-CSF. Cell lines were grown at 37 °C in a 5% CO_2 atmosphere. Media were supplemented with penicillin G (100 U/ml) and streptomycin (0.1 mg/ml). Cells were treated with the following reagents: Idarubicin (15 nM) (Sigma-Aldrich, St Louis, MO, USA), Ara-c (2.5 μM) (Sigma-Aldrich), forskolin (40 μM) (Calbiochem, San Diego, CA, USA), FTY720 (10 μM) (Calbiochem), Z-VAD-fmk (150 mM) (Promega, Madison, WI, USA) and okadaic acid (2.5 nM) (Calbiochem).

Patient samples

The study comprised BM samples of 37 patients with AML at diagnosis. All patients were treated with standard induction chemotherapy. High-dose cytarabine, and autologous or allogenic stem cell transplantation, when possible, were used as consolidation therapy. The BM samples of normal healthy donors were used as controls. This study is part of a project approved by the Comisión de Ética de Investigación Clínica (School of Medicine, University of Navarra) (037/2008). The samples used in this study were anonymous.

Nucleic acid isolation and real-time RT-PCR

Total RNA was isolated using the RNeasy minikit (Qiagen, Hilden, Germany). cDNA was synthesized with SuperScriptIII Reverse Transcriptase (Invitrogen). Quantification of the expression of SETBP1, SET, CIP2A, PP2A catalytic subunits α (PPP2CA) and β (PPP2CB), PP2A scaffold subunit PPP2R1B, and PP2A regulatory subunits PPP2R5B and PP2R5C were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), specific for each gene. Glyceraldehyde 3-phosphate dehydrogenase was used as internal control. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C_T}$ method,[40] where $\Delta\Delta C_T = (C_{T,\text{target gene}} - C_{T,GAPDH})_{\text{Cell line}} - (C_{T,\text{target gene}} - C_{T,GAPDH})_{\text{normal control}}$. A gene was considered deregulated if its expression value was higher or lower than the cutoff value established for each gene (mean + 3 s.d.), defined by the analysis of 10 normal BM samples.

Western blot analysis

Cells were lysed in 100 μl of Lysis buffer containing 1% Triton X-100 and the protease inhibitor cocktail Complete Mini (Roche Diagnostics, Mannheim, Germany). After incubation on ice (30 min), protein extracts were clarified (12 000 × *g*, 15 min, 4 °C), denatured and subjected to SDS-PAGE and western blot. Antibodies used were mouse monoclonal anti-PP2A (clone 1D6, Upstate, Temecula, CA, USA), rabbit monoclonal anti-PP2AY307 (Epitomics, Burlingame, CA, USA), rabbit polyclonal anti-Akt, rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology Inc., Beverly, MA, USA), rabbit polyclonal anti-pAkt^{Thr308} rabbit polyclonal anti-pERK1/2$^{Thr202/Tyr204}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-β-actin (Sigma), rabbit polyclonal anti-CIP2A (Novus Biologicals, Littleton, CO, USA), goat polyclonal anti-SET (Santa Cruz Biotechnology), goat monoclonal anti-PPP2R5B (Novus Biologicals) and rabbit polyclonal anti-PPP2R1B (Novus Biologicals). Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare, Piscataway, NJ, USA).

Proliferation assay and cell viability

Cell proliferation was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) and following the manufacturer's indications. Results of cell viability were confirmed by the Trypan Blue dye exclusion test.

PP2A phosphatase activity assays

PP2A assays were performed with cell lysates (50 μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore, Temecula, CA, USA) as previously described.[37]

Analysis of apoptosis and caspase activation

Caspase 3/7 activities were measured on untreated and forskolin-treated cells using the caspase Glo-3/7 assay kit (Promega). Briefly, 5×10^3 cells were plated in a white-walled 96-well plate, and the Z-DEVD reagent, the luminogenic caspase 3/7 substrate, containing a tetrapeptide Asp-Glu-Val-Asp, was added in a 1:1 ratio of reagent to sample. After 90 min at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal was measured by a FLUOstar OPTIMA luminometer (BMG Labtech,

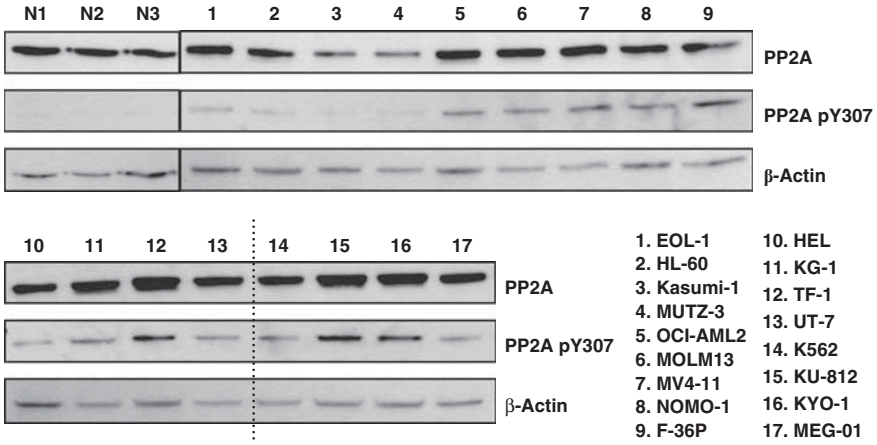


Figure 1 Analysis of PP2A activation and expression in 17 myeloid cell lines. Comparison of the PP2A-C expression and phosphorylation levels on tyrosine 307 by western blot in normal donors and in myeloid cell lines. N1-3, normal donors.

Offenburg, Germany). Differences in caspase-3/7 activity in forskolin-treated cells compared with untreated cells are expressed as fold-change in luminescence. Apoptosis was measured using the Annexin-V-FLUOS Staining Kit (Roche) and following manufacturer's instructions.

Statistical analysis

Data represented are mean of three independent experiments ± s.d. Statistical comparisons were carried out by *t*-test analysis and significance was considered when $P<0.05$. Chou–Talalay analysis was performed using the CalcuSyn Software (Biosoft, Cambridge, UK) to determine additivity between forskolin and Idarrubicin/Ara-c treatments.

Microarray analysis

RNA samples were processed following manufacturer protocols (Affymetrix, Santa Clara, CA, USA) and hybridized to the Affymetrix Human Genome-U133 Plus-2.0, which contains 54 676 probe sets (47 000 transcripts). Microarray data analysis consisted in background correction and normalization using RMA algorithm,[41] and a filtering process to eliminate low-expression probe sets. Linear Models for Microarray Data[42] was used to identify the probe sets with significant differential expression. Samples were distributed in three different groups: 'Control' including three normal control samples, 'AML' including the AML cell lines EOL-1, HL-60, Kasumi-1, OCI-AML2, MOLM13, MV4-11, HEL, KG-1, NOMO-1, F-36P and TF-1, and CML including the CML in blast crisis (BC-CML) cell lines KU-812, KYO-1, K562 and MEG-01. Data represented are the gene expression mean of each group ± s.d. Genes were selected as significant between the different groups using a B-statistic cutoff ($B>0$) or a less stringent adjusted *P*-value cutoff ($P<0.05$).

Results

PP2A is inactivated in AML cell lines

Phosphorylation of tyrosine-307 is responsible for more than 90% of the phosphatase activity of PP2A. Moreover, it has been shown that this phosphatase is inactive when tyrosine-307 is phosphorylated.[43] Thus, we assessed the phosphorylation levels on tyrosine-307 of PP2A by western blot in a panel of AML BCR/ABL-negative cell lines. A total of 11 out of 13 cell lines presented increased phosphorylation in this tyrosine. Notably, the two cell lines (Kasumi-1 and MUTZ-3) that had reduced PP2A phosphorylation were the only ones with reduced expression of the catalytic subunit (PP2A-C) (Figure 1). Four BCR/ABL-positive CML cell lines, including K562, were used as positive controls for phosphorylation on PP2A tyrosine-307.[31] To confirm whether the differences observed in PP2A protein expression in Kasumi-1 and MUTZ-3 were because of reduced transcriptional levels of PP2A, we analyzed by real-time PCR (qRT-PCR) the expression of the PP2A catalytic subunits PPP2CA and PPP2CB separately (Supplementary Table 1). We found no significant differences in PP2A-C transcriptional levels in these two cell lines, indicating that differences observed in western blot must be because of post-transcriptional regulation.

Treatment with forskolin leads to reduced proliferation that is dependent on PP2A activation

To assess whether increased PP2A activity affects cell proliferation of AML cells, KG-1 and HEL cell lines were treated with the PP2A activator forskolin or vehicle (dimethyl sulfoxide). Phosphatase assays to quantify PP2A activity levels confirmed that forskolin treatment activates PP2A: forskolin induced a 1.5- to 2-fold increase in PP2A activity (Figure 2a). To study whether a higher PP2A activity was associated with activation of PP2A protein, we pretreated KG-1 and HEL cells with the PP2A inhibitor okadaic acid for 2 h, followed by incubation with forskolin or vehicle for 48 h. Forskolin-induced PP2A activity in KG-1 and HEL cells was inhibited by okadaic acid (Figure 2a). Western blot analysis showed that similar levels of PP2Ac protein were immunoprecipitated in the PP2A phosphatase assays (Figure 2b), suggesting that forskolin-induced PP2A activity is not because of changes in PP2Ac expression levels.

We next analyzed the effect of PP2A activation on cell growth using MTS assay. We observed a decrease in the proliferation of forskolin-treated KG-1 cells compared with vehicle treated (Figure 2c). In addition, total cell counts and cell viability were confirmed with the Trypan Blue method (data not shown). These results show that PP2A activation by forskolin treatment induces toxicity in KG-1 cells. Similar results were obtained with the HEL cell line (Figure 2c). In addition, we observed that the impaired proliferation induced by forskolin was partially rescued by the treatment with the phosphatase inhibitor okadaic acid used at a concentration that inhibits PP2A but no other phosphatases.[44]

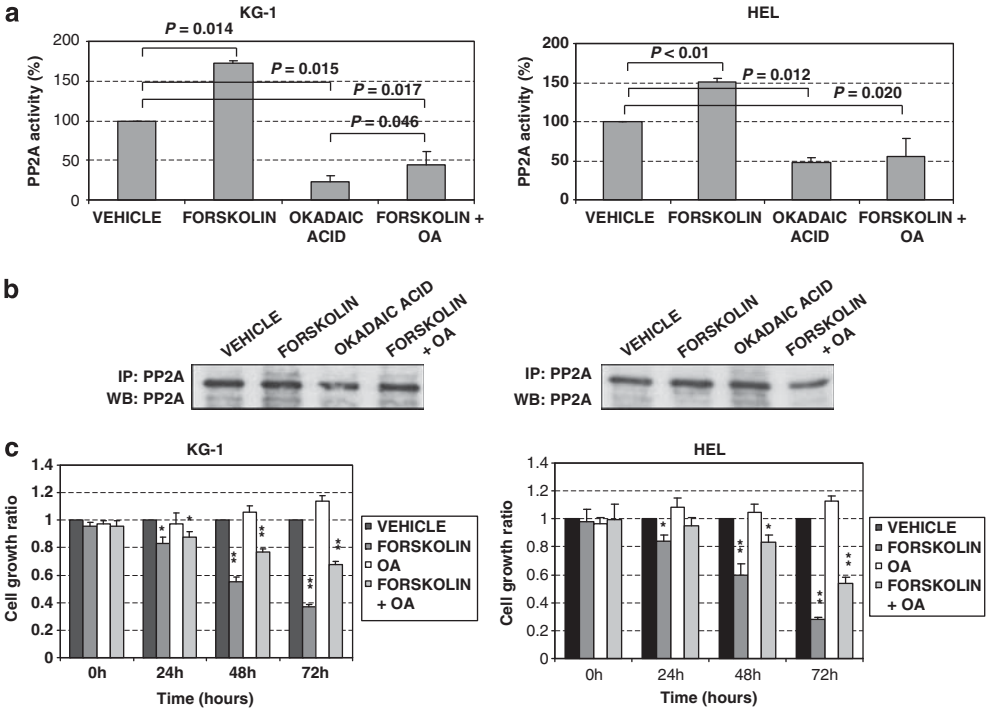


Figure 2 Forskolin treatment induces an inhibition of the proliferation that is dependent on PP2A activation. Forskolin was used at 40 μM and okadaic acid at 2.5 nM concentrations. Data represented are mean of three independent experiments ± s.d. (**a**) Forskolin-induced PP2A activity in KG-1 and HEL cell lines is inhibited by okadaic acid treatment. (**b**) Western blot analysis showing the levels of immunoprecipitated PP2A from the KG-1 and HEL lysates used in the phosphatase assays. (**c**) Inhibited proliferation induced by forskolin treatment is partially rescued by okadaic acid; $*P<0.05$; $**P<0.01$.

To confirm that the mechanism was mainly dependent on PP2A activation, we performed the same experiments in HEL and KG-1 cell lines using the PP2A-activator FTY720 observing a similar effect with this drug than with forskolin (Supplementary Figure 1). Moreover, treatment with the PP2A-activators, forskolin and FTY720, had less effect in MUTZ-3 and Kasumi-1, the cell lines with PP2A low expression, than in HEL and KG-1, suggesting that the effect of those drugs in AML cells is mainly by PP2A activation (Supplementary Figure 2).

Increased PP2A activity by forskolin induces apoptosis in AML cells

To further investigate the biological effect of the forskolin-induced PP2A activation in AML, KG-1 cells were treated with forskolin for 48 h, and we assessed apoptosis with the caspase Glo-3/7 assay kit. Vehicle-treated cells were used as controls. Consistent with its ability to enhance PP2A activity and suppress cell proliferation, forskolin had a caspase-dependent pro-apoptotic effect, increasing caspase activity by 6.5-fold in forskolin-treated KG-1 cells compared with vehicle-treated cells (Figure 3a). In addition, caspase activity in forskolin-treated cells was markedly reduced when cells were pretreated with okadaic acid or the caspase inhibitor Z-VAD-fmk. Effect in apoptosis was confirmed by an Annexin-V-based assay (Figure 3b).

Forskolin induces changes in the phosphorylation status of PP2A targets

We next analyzed by western blot the effects of the forskolin-induced PP2A activation at protein level. As expected, we observed that phosphorylation on tyrosine 307 of PP2Ac was negatively affected in cells treated with forskolin compared with cells treated with vehicle (dimethyl sulfoxide) (Figure 3c). These data confirmed the results obtained with the PP2A phosphatase assays (Figure 2a). In addition, PP2Ac phosphorylation was restored when cells were pretreated with the PP2A inhibitor okadaic acid. Consistent with previous reports about the effects of PP2A activation in myeloid BCR/ABL-positive cells,[31] forskolin treatment in AML BCR/ABL-negative cells, decreased phosphorylation (activity) of the PP2A targets AKT and ERK1/2, without affecting their expression levels. Moreover, treatment with okadaic acid rescued AKT and ERK1/2 phosphorylation in forskolin-treated KG-1 cells (Figure 3c).

Additive effect of PP2A activation with Idarubicin and Ara-c treatments in AML cells

To assess the effect of a combination between standard induction chemotherapy drugs in AML and a PP2A activator, we treated KG-1 cells with either Idarubicin or Ara-c, alone or in combination with forskolin. Of importance, we observed that PP2A activation enhanced the anti-leukemic effects mediated by both Idarubicin (Figure 4a) and Ara-c (Figure 4b) treatments in KG-1 and Hel cell lines. Moreover, Chou–Talalay analyses showed that PP2A activation has an additive anti-tumoral effect when combined with either Idarubicin or Ara-c.

PP2A inhibition is a recurrent event in AML

To further evaluate the importance of PP2A in AML, we analyzed at protein level the prevalence of PP2A inhibition in a series of 37 patients with AML at diagnosis. Patient characteristics are presented in Table 1. Increased phosphorylation of tyrosine 307 was observed in 29 out of 37 cases (78.4%) (Figure 5a and Supplementary Figure 3). In addition,

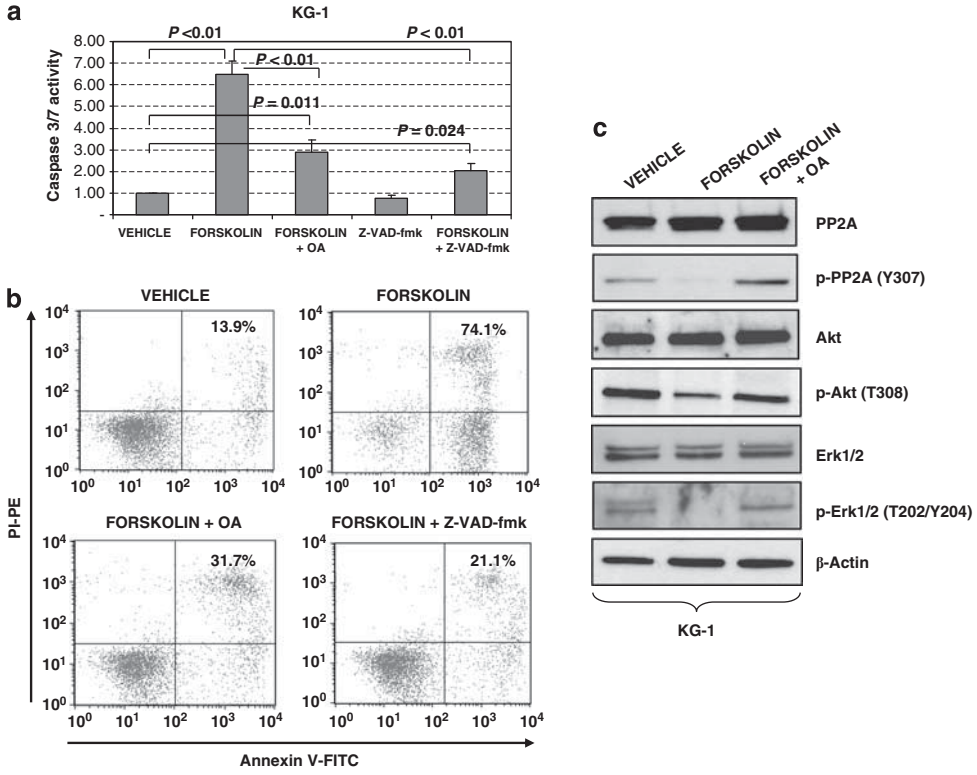


Figure 3 Forskolin induces caspase-dependent apoptosis together with changes in the phosphorylation status of PP2A targets. Forskolin was used at 40 μM and okadaic acid at 2.5 nM concentrations. Z-VAD-fmk was used at 150 mM. Data represented are mean of three independent experiments ± s.d. (**a**) Caspase 3/7 assays in untreated, forskolin-treated, forskolin/okadaic acid-treated, Z-VAD-fmk-treated and forskolin/Z-VAD-fmk-treated KG-1 cells. (**b**) Annexin-V/propidium iodide assays in untreated, forskolin-treated, forskolin/okadaic acid-treated and forskolin/Z-VAD-fmk-treated KG-1 cells. (**c**) Western blot showing the effect of forskolin and forskolin/okadaic acid treatments in KG-1 cells on PP2A, AKT and ERK1/2 activity and expression.

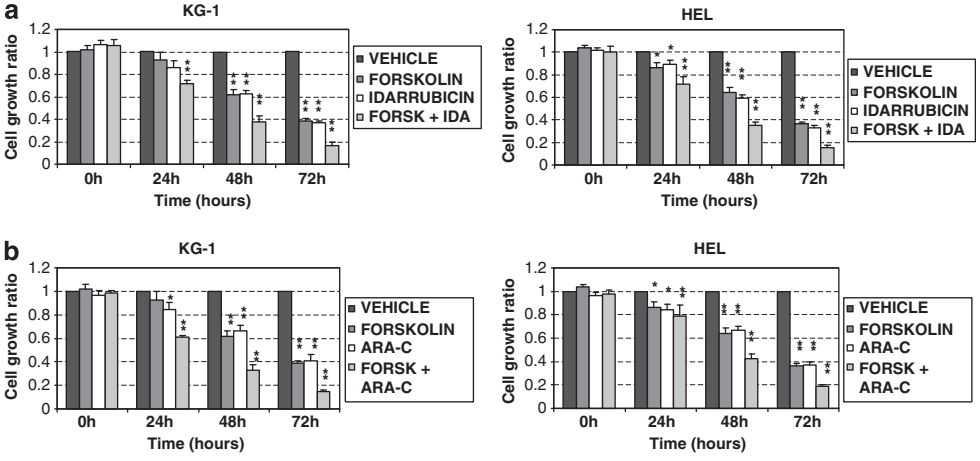


Figure 4 PP2A activation boosts anti-leukemic effects of Idarubicin and Ara-c treatments. Data represented are mean of three independent experiments ± s.d.; **P*<0.05; ***P*<0.01; (**a**) MTS assays showing the effect of PP2A activation by forskolin (40 μM) on the cell growth ratio of KG-1 and HEL cells treated with Idarubicin (15 nM). (**b**) MTS assays showing the effect of PP2A activation by forskolin (40 μM) on the cell growth ratio of KG-1 and HEL cells treated with Ara-c (2.5 μM). Cells treated with vehicle (DMSO) were used as controls.

PP2A activity was compared between samples of eight AML patients and three normal controls, and we observed a significant reduction of PP2A activity in all the eight patient samples analyzed (Supplementary Figure 4A). These results would indicate that PP2A inhibition is a recurrent event in AML. Moreover, we analyzed samples of three patients at diagnosis, complete remission and relapse, observing that PP2A phosphorylation decreases at complete remission and increases at relapse (Figure 5b and Supplementary Figure 4B).

To investigate the possible causes of PP2A phosphorylation, we analyzed the expression of SET, SETBP1 and CIP2A, and the presence of the constitutive-activating mutation JAK2-V617F in this series of patients. We found overexpression of SET and/or SETBP1 in 55% cases with increased PP2A phosphorylation

Table 1 Clinical and molecular characteristics of a series of 37 patients with AML

	No. (%)
Sex	
Male	22 (59.5)
Female	15 (40.5)
Age	
⩽60 years	12 (33.3)
>60 years	24 (66.7)
Complete remission	
No	9 (32.2)
Yes	19 (67.8)
Diagnosis	
AML-M0	3 (8.2)
AML-M1	7 (18.8)
AML-M2	5 (13.6)
AML-M4	9 (24.3)
AML-M5	9 (24.3)
AML-M6	2 (5.4)
AML-M7	2 (5.4)
sAML	
No	31 (83.8)
Yes	6 (16.2)
Cytogenetic group	
Good	2 (5.4)
Intermediate	29 (78.4)
Poor	6 (16.2)
SETBP1 overexpression	
No	25 (67.6)
Yes	12 (32.4)
SET overexpression	
No	26 (70.3)
Yes	11 (29.7)
JAK2 (V617F)	
No	31 (88.6)
Yes	4 (11.4)
FLT3-ITD	
No	29 (82.8)
Yes	6 (17.2)
NPM1 mutated	
No	18 (62.2)
Yes	11 (37.8)
WT1 overexpression	
No	8 (23.6)
Yes	26 (76.4)
CIP2A overexpression	
No	27 (90)
Yes	3 (10)

Abbreviation: AML, acute myeloid leukemia.

(16/29), and in none of the cases with low phosphorylated levels (Table 2). CIP2A overexpression was detected only in two cases with high phosphorylation. Analysis by western blot confirmed CIP2A and SET overexpression also at the protein level (Supplementary Figure 5). In addition, JAK2-V617F was detected in one of the patients with high PP2A phosphorylation, although also in three cases with low PP2A phosphorylation (Supplementary Table 2). As detected in the cell lines, six out of eight patient samples with low PP2A phosphorylation had a reduced expression of PP2A-C (Figure 5a). Interestingly, two patients had both PP2A inhibition and FLT3, constitutively activated by the FLT3-ITD mutation (Supplementary Table 2), two aberrations that led to the activation of the transduction pathways JAK/STAT, ERK and AKT.[45]

Genome-wide gene expression analysis of PP2A subunits in myeloid cell lines

The fact that 12 of the patients included in our series had no known mechanism that could explain PP2A inactivation prompted us to perform expression arrays of 16 myeloid cell lines to obtain an overview of the expression of the PP2A subunits. One of these cases had a deletion del(11)(q13q23), a region in which the locus of the PP2A scaffold subunit PP2A-Aβ (PPP2R1B) (11q23) and the regulatory subunit PPP2R5B (11q13) are located. We hypothesized that as previously described,[9] downregulation of some scaffold and regulatory subunits could affect the activity of PP2A.

Expression arrays of the cell lines showed a significant downregulation of the regulatory subunits PPP2R5B and PPP2R5C in both AML and BC-CML cell lines when compared with normal controls; interestingly, PPP2R5B was significantly more downregulated in AML than in CML (Supplementary Figure 6). Data were validated by qRT-PCR (Supplementary Table 1). Moreover, analysis of the patient samples revealed that PP2A-Aβ (PPP2R1B), PPP2R5B or/and PPP2R5C were downregulated in several AML patient samples (Supplementary Table 3). We could analyze at protein level 16 cases that had PPP2R5B downregulation by real time RT-PCR (qRT-PCR), and 15 out of 16 had decreased PPP2R5B protein expression. Moreover, three cases with no PPP2R5B downregulation (P17-19) were included in the study and had normal PPP2R5B protein levels (Supplementary Figure 7A). We could not perform the study of PPP2R5C at protein level. With regard to PPP2R1B, there was no good correlation between mRNA and protein: only 8 out of the 17 cases analyzed had decreased PPP2R1B protein levels, the other 9 were normal. Moreover, we included in the study five cases with no PPP2R1B downregulation by qRT-PCR: four had normal PPP2R1B protein levels (P6, P13, P17, P19), and one case had PPP2R1B low (Supplementary Figure 7B). Taken together, these results show that downregulation of these subunits is a common event in AML that could contribute to PP2A inactivation.

Discussion

PP2A is a human tumor suppressor that inhibits cellular transformation by regulating the activity of several signaling proteins critical for malignant cell behavior. We report here that PP2A inhibition is a recurrent event that could have an important role in AML. We demonstrate that PP2A activation by forskolin induce growth inhibition, caspase-dependent apoptosis, and the modification of downstream targets such as AKT and ERK1/2. Of importance, our data provides evidence that PP2A activation could be a promising therapeutic target in combination with drugs used in standard induction therapy, such as Idarubicin and Ara-c.

It has been reported that impaired PP2A activity has a key role in BCR/ABL-positive leukemias, such as CML and acute lymphoblastic leukemia,[31,32] in myeloid precursors expressing imatinib-sensitive (V560G) and imatinib-resistant (D816 V) mutant c-KIT,[36] and also in chronic lymphocytic leukemia;[46] moreover, activation of PP2A by either FTY720 or forskolin seems to have promising therapeutic effects in these diseases.

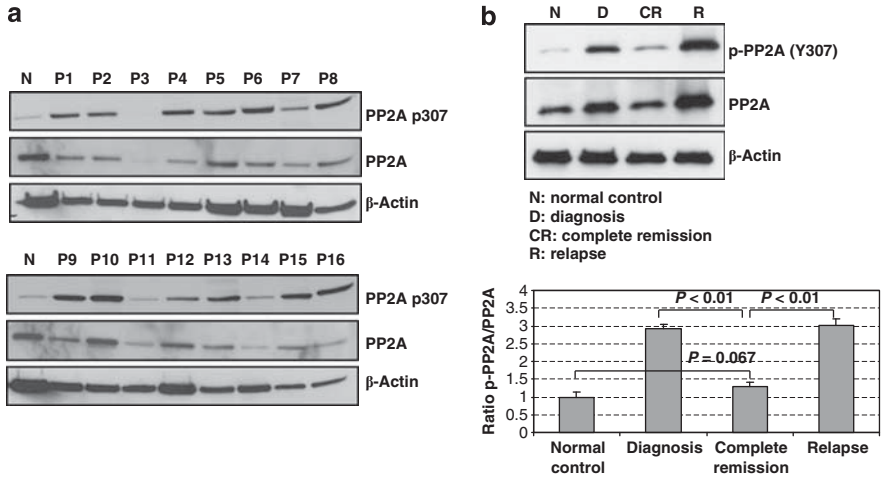


Figure 5 Comparison by western blot of the PP2Ac expression and activity levels between patient samples and normal donors. (**a**) Analysis of PP2A in 16 samples of AML patients at diagnosis. (**b**) Comparison of PP2A activation in samples of a patient with AML at diagnosis, complete remission and relapse, including a densitometric analysis of the p-PP2A/PP2A ratio.

Table 2 Overexpression of SET and SETBP1, and presence of JAK2-V617F in a series of 37 patients with AML

	PP2A phosphorylated	*PP2A not phosphorylated*	*Total*
SET overexpression	4	0	4
SETBP1 overexpression	5	0	5
SET and SETBP1 overexpression	7	0	7
JAK2-V617F	1	3	4
CIP2A overexpression	2	1	3
TOTAL	29	8	37

Abbreviation: AML, acute myeloid leukemia.

Many kinases have been reported to be deregulated in AML; however, the role of phosphatases in the cellular transformation of this disease remains underexplored.[10] As indicated above, only few studies have reported reduced PP2A activity in AML.[35,36] Our group has recently reported an impaired PP2A activity by SET as a consequence of SETBP1 overexpression, a recurrent event in AML (27%).[37] This leads us to hypothesize that PP2A inhibition could be a recurrent event in AML. Our results in both cell lines and patient samples confirm this hypothesis and show that PP2A inhibition has an important role in AML transformation, as the pharmacological activation of PP2A *in vitro* reverse some of the leukemogenic features (Figures 2 and 3; Supplementary Figures 1 and 2). When we investigated the PP2A status at protein level in 37 patients with AML at diagnosis, we observed PP2A inhibition in 78% of cases. Interestingly, we detected reduced PP2Ac expression in six out of eight patient samples with no PP2A phosphorylation; this observation is consistent with the reduced PP2Ac levels observed in Kasumi-1 and MUTZ-3, and could suggest that a reduced protein expression could be a mechanism to decrease PP2A activity in AML. Furthermore, we also observed an additive effect of PP2A activation by forskolin with the anti-leukemic effects, induced by Idarubicin or Ara-c, in both KG-1 and HEL cells, suggesting that PP2A activation could be a new alternative for treating AML in combination with standard induction chemotherapy (Figure 4). It has been reported that forskolin-induced effects on cell growth and apoptosis of AML cells at the concentrations used in this study do not impair viability of normal BM cells;[31] this observation would support the use of PP2A activators in future therapies for patients with AML. Moreover, we show that forskolin suppresses Akt and ERK1/2 function in an okadaic acid sensitive manner, indicating that its action in AML is dependent on PP2A activation (Figure 3). These results are also interesting, as several PP2A targets have been reported to be deregulated in AML and some of them, as AKT, have been associated to poor outcome.[35] Our results support the premise that PP2A inactivation might be one of the events that contribute to these alterations, as PP2A has an integral role in the regulation of a number of major signaling pathways whose deregulation can contribute to cancer.[9]

Although evidence suggested that PP2A might be a tumor suppressor protein, recent findings provide convincing evidence that suppression of PP2A activity cooperates with other oncogenic changes to cause transformation of multiple cell types.[3,23,47] In our series, we found that 34% of patients with PP2A inactivated, (10/29) had either FLT3 and/or NPM1 mutated (Supplementary Table 2). Interestingly, the constitutive activation of FLT3 in cases with FLT3-ITD also activates both Akt and ERK1/2,[48] suggesting that in some patients PP2A inactivation and FLT3-ITD could cooperate in the transformation of AML.

PP2A can be inhibited by the small-tumor antigen of DNA tumor viruses, by upregulation of endogenous PP2A inhibitors, through mutational inactivation of the structural subunits, or by decreased expression of either the scaffold or the regulatory subunits.[9,10] On the other hand, JAK2 constitutive activity[49,50] and SETBP1 overexpression in BCR/ABL-negative AML[37] might independently contribute to PP2A inactivation. Our results show that the mechanisms of PP2A inactivation in AML might be the overexpression of the physiological PP2A inhibitors CIP2A[33] and SET,[51] the overexpression of SETBP1, or the downregulation of PP2A subunits, suggesting that dysfunction of several distinct PP2A complexes may contribute to cell transformation. Overexpression of CIP2A, SET or SETBP1 could explain the mechanism of PP2A inactivation in 58% of our cases (17/29). SET is upregulated in multiple solid tumors,[52] and has been reported to be fused to NUP214/CAN in a patient with AML.[53] Importantly, Neviani *et al.*[31] demonstrated that PP2A inactivation in CML-BC results from increased expression of SET, which is induced by BCR/ABL in a dose- and kinase-dependent manner and, that like BCR/ABL, SET progressively increases during

transition to blast crisis. In fact, imatinib treatment and SET downregulation restored PP2A activity back to normal levels. Our results show that high expression of SET also leads to PP2A inactivation in AML, independently of BCR/ABL induction. In addition, the activating mutation JAK2-V617F was detected in one of the samples analyzed; however, three cases with JAK2-V617F had no PP2A hyperphosphorylation, suggesting that either this mutation would need other additional changes to inactivate PP2A or that JAK2-V617F is not causal in activating PP2A in AML.

As in 12 cases, the mechanism remained undetermined, and one case had a deletion del(11)(q13q23), a region in which the loci of the PP2A scaffold subunit PP2A-Aβ (PPP2R1B) (11q23) and the regulatory subunit PPP2R5B (11q13) are located, we hypothesized that downregulation of some subunits could affect the activity of PP2A, as previously reported.[9] Analysis of 16 myeloid cell lines and AML patient samples showed genetic aberrations affecting PP2A subunits that could be having an important role in the PP2A inhibition observed in AML. We found downregulation of PPP2R5B and PPP2R5C in both AML and CB-CML cell lines (Supplementary Figure 6). It has been reported that PPP2R5B is a tumor suppressor that negatively regulates Pim-1 protein kinase, which is known to enhance the ability of c-Myc to induce lymphomas.[26] Furthermore, it has been described that suppression of PPP2R5C expression contributes to the experimental transformation of human cells.[28] Our data suggest that loss of PPP2R5B and PPP2R5C could be having a role in AML development, contributing to deregulate the correct PP2A function. We also found that downregulation of the Aβ subunit is a common event in AML. Most cellular PP2A holoenzymes contain the Aα isoform of the scaffold subunit, but a small fraction (10%) contain a second isoform-termed Aβ. Although mutations that disrupt the ability of Aβ to form holoenzymes *in vitro* were identified in several types of cancer,[12,54,55] the report by Sablina *et al*.[21] provides the first hard evidence that loss of functional Aβ caused by these cancer-associated mutations contribute to transformation.[3] Further studies are necessary to clarify the importance of the downregulation of these PP2A subunits in AML.

Taking together, our results suggest that functional inactivation of the PP2A tumor suppressor is a recurrent event that seems to represent an important mechanism in the leukemogenic transformation of AML. We show that functional loss of PP2A activity could occur through different contributing mechanisms such as enhancement of endogenous PP2A inhibitors, decreased PP2A expression of the structural or regulatory subunits of PP2A. Moreover, although PP2A activators are not still clinically available, the results obtained in this study suggest that PP2A activation could be considered as a future therapeutic alternative for AML. The knowledge that pharmacological restoration of PP2A activity is able to antagonize leukemogenesis and has an additive effect with other drugs used in the treatment of AML highlights PP2A as a potential target for future therapies combined with PP2A activators.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Ministerio Educación y Ciencia (SAF2005/06425 and AP2006-03038), Ministerio Ciencia e Innovación (PI081687), Departamento Salud del Gobierno de Navarra (14/2008), ISCIII-RTICC (RD06/0020/0078) and Fundación para la Investigación Médica Aplicada y UTE (Spain). We thank Dr Anna Sablina for useful discussion, Elizabeth Guruceaga of the Unit of Proteomics, Genomics and Bioinformatics of CIMA for bioinformatics analysis, and Leyre Urquiza and Marisol Gonzalez-Huarriz for technical assistance.

References

1 Rowe JM. Optimal induction and post-remission therapy for AML in first remission. *Hematology Am Soc Hematol Educ Program* 2009; **1**: 396–405.
2 Haferlach T. Molecular genetic pathways as therapeutic targets in acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program* 2008; **1**: 400–411.
3 Mumby M. PP2A: unveiling a reluctant tumor suppressor. *Cell* 2007; **130**: 21–24.
4 Millward TA, Zolnierowicz S, Hemmings BA. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem Sci* 1999; **24**: 186–191.
5 Hemmings BA, Adams-Pearson C, Maurer F, Muller P, Goris J, Merlevede W *et al*. α- and β-Forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry* 1990; **29**: 3166–3173.
6 Zhou J, Pham HT, Ruediger R, Walter G. Characterization of the Aα and Aβ subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. *Biochem J* 2003; **369**: 387–398.
7 Arino J, Woon CW, Brautigan DL, Miller Jr TB, Johnson GL. Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. *Proc Natl Acad Sci USA* 1988; **85**: 4252–4256.
8 Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem* 1989; **58**: 453–508.
9 Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta* 2009; **1795**: 1–15.
10 Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* 2008; **14**: 152–160.
11 Ruediger R, Pham HT, Walter G. Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. *Oncogene* 2001; **20**: 10–15.
12 Ruediger R, Pham HT, Walter G. Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. *Oncogene* 2001; **20**: 1892–1899.
13 Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J *et al*. Alterations of the PPP2R1B gene in human lung and colon cancer. *Science* 1998; **282**: 284–287.
14 Calin GA, di Iasio MG, Caprini E, Vorechovsky I, Natali PG, Sozzi G *et al*. Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms. *Oncogene* 2000; **19**: 1191–1195.
15 Esplin ED, Ramos P, Martinez B, Tomlinson GE, Mumby MC, Evans GA. The glycine 90 to aspartate alteration in the Abeta subunit of PP2A (PPP2R1B) associates with breast cancer and causes a deficit in protein function. *Genes Chromosomes Cancer* 2006; **45**: 182–190.
16 Takagi Y, Futamura M, Yamaguchi K, Aoki S, Takahashi T, Saji S. Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. *Gut* 2000; **47**: 268–271.
17 Tamaki M, Goi T, Hirono Y, Katayama K, Yamaguchi A. PPP2R1B gene alterations inhibit interaction of PP2A-Abeta and PP2A-C proteins in colorectal cancers. *Oncol Rep* 2004; **11**: 655–659.
18 Kalla C, Scheuermann MO, Kube I, Schlotter M, Mertens D, Dohner H *et al*. Analysis of 11q22-q23 deletion target genes in B-cell chronic lymphocytic leukaemia: evidence for a pathogenic role of NPAT, CUL5, and PPP2R1B. *Eur J Cancer* 2007; **43**: 1328–1335.

19 Hemmer S, Wasenius VM, Haglund C, Zhu Y, Knuutila S, Franssila K *et al*. Alterations in the suppressor gene PPP2R1B in parathyroid hyperplasias and adenomas. *Cancer Genet Cytogenet* 2002; **134**: 13–17.
20 Chou HC, Chen CH, Lee HS, Lee CZ, Huang GT, Yang PM *et al*. Alterations of tumour suppressor gene PPP2R1B in hepatocellular carcinoma. *Cancer Lett* 2007; **253**: 138–143.
21 Sablina AA, Chen W, Arroyo JD, Corral L, Hector M, Bulmer SE *et al*. The tumor suppressor PP2A Abeta regulates the RalA GTPase. *Cell* 2007; **129**: 969–982.
22 Chen W, Arroyo JD, Timmons JC, Possemato R, Hahn WC. Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. *Cancer Res* 2005; **65**: 8183–8192.
23 Sablina AA, Hahn WC. The role of PP2A A subunits in tumor suppression. *Cell Adh Migr* 2007; **1**: 140–141.
24 Arnold HK, Sears RC. Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. *Mol Cell Biol* 2006; **26**: 2832–2844.
25 Margolis SS, Perry JA, Forester CM, Nutt LK, Guo Y, Jardim MJ *et al*. Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell* 2006; **127**: 759–773.
26 Ma J, Arnold HK, Lilly MB, Sears RC, Kraft AS. Negative regulation of Pim-1 protein kinase levels by the B56beta subunit of PP2A. *Oncogene* 2007; **26**: 5145–5153.
27 Letourneux C, Rocher G, Porteu F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *EMBO J* 2006; **25**: 727–738.
28 Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* 2004; **5**: 127–136.
29 Shouse GP, Nobumori Y, Liu X. A B56gamma mutation in lung cancer disrupts the p53-dependent tumor-suppressor function of protein phosphatase 2A. *Oncogene* 2010; **29**: 1669–1681.
30 Li HH, Cai X, Shouse GP, Piluso LG, Liu X. A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J* 2007; **26**: 402–411.
31 Neviani P, Santhanam R, Trotta R, Notari M, Blaser BW, Liu S *et al*. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell* 2005; **8**: 355–368.
32 Neviani P, Santhanam R, Oaks JJ, Eiring AM, Notari M, Blaser BW *et al*. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest* 2007; **117**: 2408–2421.
33 Junttila MR, Puustinen P, Niemela M, Ahola R, Arnold H, Bottzauw T *et al*. CIP2A inhibits PP2A in human malignancies. *Cell* 2007; **130**: 51–62.
34 Yokoyama N, Reich NC, Miller WT. Determinants for the interaction between Janus kinase 2 and protein phosphatase 2A. *Arch Biochem Biophys* 2003; **417**: 87–95.
35 Gallay N, Dos Santos C, Cuzin L, Bousquet M, Simmonet Gouy V, Chaussade C *et al*. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* 2009; **23**: 1029–1038.
36 Roberts KG, Smith AM, McDougall F, Carpenter H, Horan M, Neviani P *et al*. Essential requirement for PP2A inhibition by the oncogenic receptor c-KIT suggests PP2A reactivation as a strategy to treat c-KIT+ cancers. *Cancer Res* 2010; **70**: 5438–5447.
37 Cristobal I, Blanco FJ, Garcia-Orti L, Marcotegui N, Vicente C, Rifon J *et al*. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood* 2010; **115**: 615–625.
38 Luo LY, Huang J, Gou BD, Zhang TL, Wang K. Induction of human promyelocytic leukemia HL-60 cell differentiation into monocytes by arsenic sulphide: involvement of serine/threonine protein phosphatases. *Leuk Res* 2006; **30**: 1399–1405.
39 Martelli AM, Papa V, Tazzari PL, Ricci F, Evangelisti C, Chiarini F *et al*. Erucylphosphohomocholine, the first intravenously applicable alkylphosphocholine, is cytotoxic to acute myelogenous leukemia cells through JNK- and PP2A-dependent mechanisms. *Leukemia* 2010; **24**: 687–698.
40 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001; **25**: 402–408.
41 Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of affymetrix genechip probe level data. *Nucleic Acids Res* 2003; **31**: e15.
42 Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article 3.
43 Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science* 1992; **257**: 1261–1264.
44 Saydam G, Aydin HH, Sahin F, Selvi N, Oktem G, Terzioglu E *et al*. Involvement of protein phosphatase 2A in interferon-alpha-2b-induced apoptosis in K562 human chronic myelogenous leukaemia cells. *Leuk Res* 2003; **27**: 709–717.
45 Gilliland DG, Griffin JD. Role of FLT3 in leukemia. *Curr Opin Hematol* 2002; **9**: 274–281.
46 Liu Q, Zhao X, Frissora F, Ma Y, Santhanam R, Jarjoura D *et al*. FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. *Blood* 2008; **111**: 275–284.
47 Junttila MR, Westermarck J. Mechanisms of MYC stabilization in human malignancies. *Cell Cycle* 2008; **7**: 592–596.
48 Kornblau SM, Singh N, Qiu Y, Chen W, Zhang N, Coombes KR. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. *Clin Cancer Res* 2010; **16**: 1865–1874.
49 Samanta AK, Chakraborty SN, Wang Y, Kantarjian H, Sun X, Hood J *et al*. Jak2 inhibition deactivates Lyn kinase through the SET-PP2A-SHP1 pathway, causing apoptosis in drug-resistant cells from chronic myelogenous leukemia patients. *Oncogene* 2009; **28**: 1669–1681.
50 Yokoyama N, Reich NC, Miller WT. Involvement of protein phosphatase 2A in the interleukin-3-stimulated Jak2-Stat5 signaling pathway. *J Interferon Cytokine Res* 2001; **21**: 369–378.
51 Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* 1996; **271**: 11059–11062.
52 Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. *J Biol Chem* 2002; **277**: 25026–25031.
53 Rosati R, La Starza R, Barba G, Gorello P, Pierini V, Matteucci C *et al*. Cryptic chromosome 9q34 deletion generates TAF-Ialpha/CAN and TAF-Ibeta/CAN fusion transcripts in acute myeloid leukemia. *Haematologica* 2007; **92**: 232–235.
54 Xu Y, Xing Y, Chen Y, Chao Y, Lin Z, Fan E *et al*. Structure of the protein phosphatase 2A holoenzyme. *Cell* 2006; **127**: 1239–1251.
55 Cho US, Xu W. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. *Nature* 2007; **445**: 53–57.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Supplementary Table 1 Quantification of the expression of PP2A-Cα (PPP2CA), P PP2A-Cβ (PPP2CB), PP2A-Aβ (PPP2R1B), PPP2R5B and PPP2R5C by real-time PCR in 17 myeloid cell lines. $2^{-\Delta\Delta C}{}_{T}$ represents normalized gene amount in a cell line relative to normal controls.

Cell line	$2^{-\Delta\Delta C}{}_{T}$ (PPP2CA)	$2^{-\Delta\Delta C}{}_{T}$ (PPP2CB)	$2^{-\Delta\Delta C}{}_{T}$ (PPP2R1B)	$2^{-\Delta\Delta C}{}_{T}$ (PPP2R5B)	$2^{-\Delta\Delta C}{}_{T}$ (PPP2R5C)
EOL-1	1.29	0.98	0.41	0.09	0.78
HL-60	0.82	0.48	0.23	0.05	0.63
Kasumi-1	1.18	0.48	0.28	0.05	0.26
MUTZ-3	1.39	0.43	0.50	0.06	0.46
OCI-AML2	0.72	0.55	0.34	0.08	0.54
MOLM13	1.03	0.77	0.22	0.03	0.39
MV4-11	2.19	0.82	0.38	0.08	0.91
NOMO-1	0.83	1.24	0.46	0.04	0.56
F-36P	3.84	3.61	0.27	0.13	0.49
HEL	1.01	1.21	0.34	0.07	0.43
KG-1	0.85	0.42	0.72	0.09	0.59
TF-1	2.17	1.01	0.77	0.10	1.01
UT-7	1.77	1.72	0.60	0.18	0.96
K562	1.80	2.51	0.59	0.28	0.74
KU-812	1.91	2.39	0.54	0.36	1.23
KYO-1	3.14	2.77	0.63	0.77	0.56
MEG-01	3.39	1.80	0.50	0.42	0.61

Cut-off (downregulation): PPP2CA: 0.63; PPP2CB: 0.60; PPP2R1B: 0.67; PPP2R5B: 0.39; PPP2R5C: 0.65

Supplementary Table 2 Clinical and molecular characteristics of the 37 patients with AML included in the study.

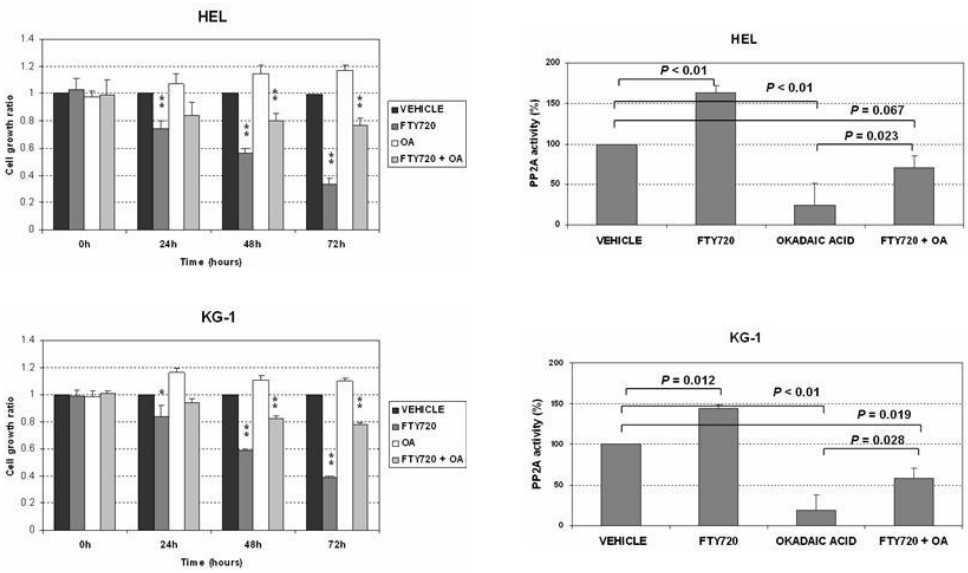
Case	Sex	Age	FAB classification	AML type	Karyotype	PP2A hyper-phosphorylation	PP2Ac protein expression	SET OE	SETBP1 OE	CIP2A OE	JAK2-V617F	PPP2R1B DR	PPP2R5B DR	PPP2R5C DR	FLT3-ITD	NPM1 mutated
17695	M	56	AML-M2	de novo	47,XY,+8	no	reduced	no	no	yes	yes	yes	no	yes	no	no
19608	F	76	AML-M2	sAML	46,XX	no	reduced	no	no	ND	yes	yes	yes	yes	no	no
24860	F	42	AML-M5	de novo	48,XX,del(1)(p21),+4,+6,del(11)(q21),der(11)add(11)(p15)	no	reduced	no	no	no	no	yes	ND	ND	no	no
22271	M	66	AML-M1	sAML	46,XY	no	reduced	no	no	no	yes	yes	yes	no	no	no
19820	F	46	AML-M4	de novo	46,XX	no	normal	no	no	ND	no	ND	ND	ND	yes	ND
21507	F	53	AML-M4	de novo	46,XX	no	reduced	no	no	no	no	yes	yes	yes	no	yes
25222	F	64	AML-M5	de novo	46,XX	no	reduced	no	no	no	no	yes	yes	yes	no	yes
26164	M	72	AML-M5	de novo	47,XY,der(3),+8	no	normal	no	no	no	no	ND	ND	ND	no	ND
22892	M	68	AML-M2	sAML	46,XY	yes	reduced	no	no	no	yes	yes	no	no	yes	no
15619	M	74	AML-M5	de novo	46,XY	yes	normal	yes	no	no	no	yes	yes	yes	no	ND
19937	M	43	AML-M5	de novo	46,XY	yes	normal	yes	no	ND	no	yes	no	yes	no	yes
24415	M	73	AML-M4	de novo	46,XY	yes	reduced	yes	no	no	no	yes	ND	no	no	yes

Case	Sex	Age	FAB classification	AML type	Karyotype	PP2A hyper-phosphorylation	PP2Ac protein expression	SET OE	SETBP1 OE	CIP2A OE	JAK2-V617F	PPP2R1B DR	PPP2R5B DR	PPP2R5C DR	FLT3-ITD	NPM1 mutated
27578	M	71	AML-M1	de novo	46,XY	yes	normal	yes	no	no	no	yes	yes	yes	yes	yes
18154	M	ND	AML-M0	de novo	46,XY	yes	normal	no	yes	yes	no	no	no	no	no	no
19449	M	76	AML-M1	de novo	46,XY/47,XY,+13	yes	normal	no	yes	ND	no	no	no	ND	no	no
24212	F	59	AML-M5	de novo	46,XX	yes	reduced	no	yes	no	no	yes	no	yes	no	no
25448	F	69	AML-M7	de novo	46,XX,add(3)(q?)	yes	normal	no	yes	no	no	yes	yes	yes	no	no
32014	M	76	AML-M6	de novo	46,XY,t(12;18)(p13;q22)/47,XY,idem,+19	yes	normal	no	yes	no	ND	yes	yes	yes	ND	no
14412	F	61	AML-M4	de novo	46,XX	yes	normal	yes	yes	ND	no	no	ND	yes	yes	yes
18425	M	64	AML-M1	de novo	45,XY,-7,del(11)(q23),-14,-17,-21,+3mar/88,XXXX,cx	yes	normal	yes	yes	no	no	yes	no	no	no	no
25297	F	69	AML-M7	de novo	46,XX/46,XX,add(3)(q?)	yes	normal	yes	yes	no	ND	no	no	no	ND	ND
27101	M	40	AML-M4	de novo	ND	yes	normal	yes	yes	yes	no	no	no	no	no	no
27298	F	67	AML-M1	de novo	46,XX	yes	reduced	yes	yes	no	no	yes	no	no	yes	yes
27990	M	41	AML-M4	de novo	ND	yes	normal	yes	yes	no	no	no	no	no	no	no
28685	M	75	AML-M0	de novo	46,XY,t(11;12)(q13;p13),der(12),del(17)(p13),der(17)	yes	reduced	yes	yes	ND	no	yes	no	no	no	ND
17393	M	67	AML-M5	de novo	46,XY	yes	reduced	no	no	no	no	no	yes	yes	no	yes

Case	Sex	Age	FAB classification	AML type	Karyotype	PP2A hyper-phosphorylation	PP2Ac protein expression	SET OE	SETBP1 OE	CIP2A OE	JAK2-V617F	PPP2R1B DR	PPP2R5B DR	PPP2R5C DR	FLT3-ITD	NPM1 mutated
17619	F	65	AML-M4	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	no	yes
18937	M	6	AML-M4	de novo	46,XY/46,XY,inv(7)(p21q36),del(11)(q13q23)	yes	reduced	no	no	ND	no	yes	yes	no	no	ND
20762	F	73	AML-M4	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	yes	yes
21720	F	55	AML-M0	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	no	no
23816	F	64	AML-M5	sAML	46,XX	yes	reduced	no	no	no	no	yes	yes	yes	no	yes
24043	M	71	AML-M1	sAML	46,XY,+8	yes	normal	no	no	no	no	yes	yes	yes	no	no
24731	M	55	AML-M1	de novo	46,XY	yes	normal	no	no	no	no	yes	yes	no	no	no
24756	F	59	AML-M2	de novo	46,XX	yes	normal	no	no	no	no	yes	yes	yes	no	no
26916	M	64	AML-M2	de novo	47,XY,+8	yes	normal	no	no	no	no	yes	yes	yes	no	ND
27535	M	71	AML-M6	sAML	47,XY,del(20)(q11)x2	yes	normal	no	no	no	no	yes	yes	yes	no	ND
28827	M	75	AML-M5	de novo	47,XY,+19	yes	reduced	no	no	no	no	yes	no	yes	no	no

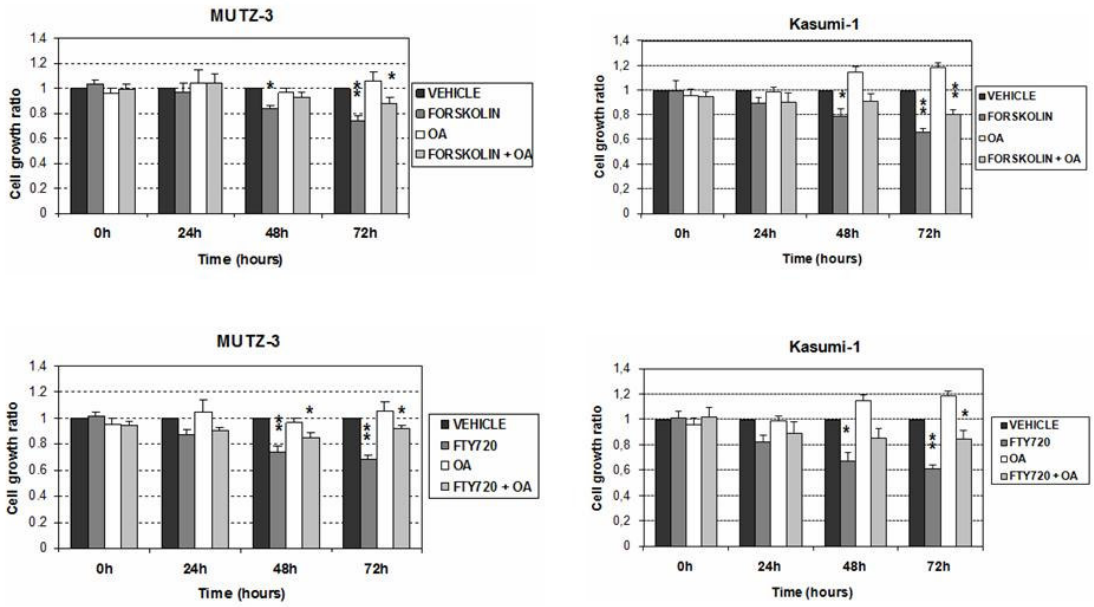
Supplementary Table 3 Downregulation of PP2A-Aβ (PPP2R1B), PPP2R5B and PPP2R5C in a series of 37 patients with AML.

	Total number of cases (No. 37)	PP2A phosphorylated	PP2A not phosphorylated
PP2A-Aβ (PPP2R1B)			
No downregulation	7 (20%)	7 (24.2%)	0
Downregulation	28 (80%)	22 (75.8%)	6 (100%)
PPP2R5B			
No downregulation	13 (41.6%)	11 (42.3%)	2 (33.3%)
Downregulation	19 (59.4%)	15 (57.7%)	4 (66.7%)
PPP2R5C			
No downregulation	12 (36.4%)	11 (39.3%)	1 (20%)
Downregulation	21 (63.6%)	17 (60.7%)	4 (80%)



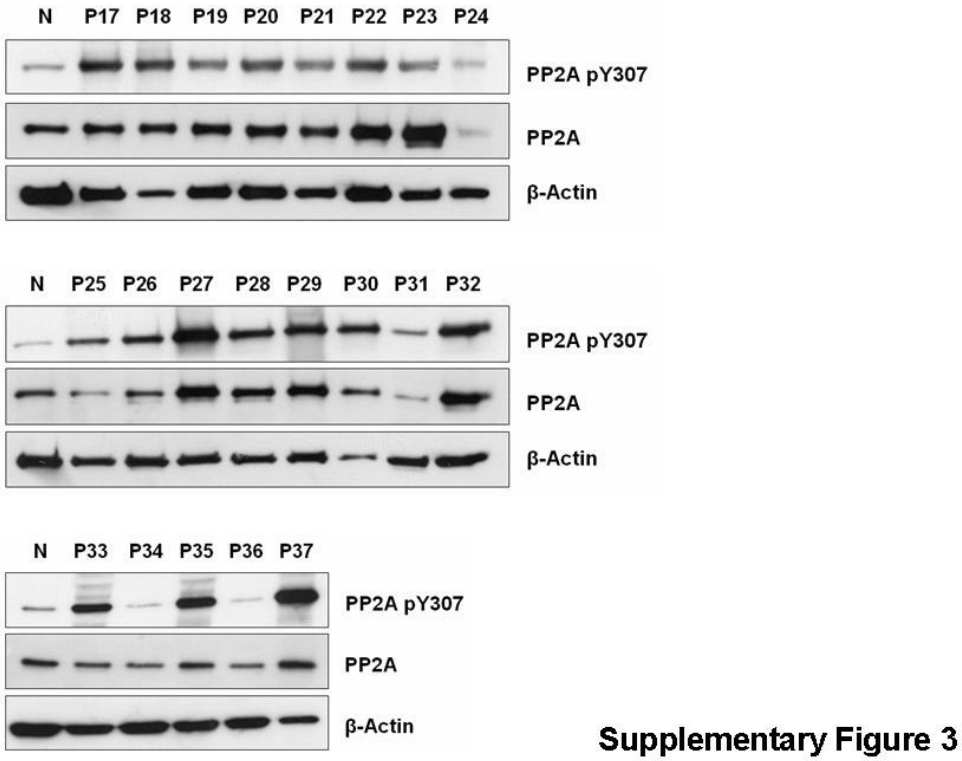
Supplementary Figure 1

Supplementary Figure 1 FTY720-induced PP2A activity and impaired proliferation in KG-1 and HEL cell lines is inhibited by okadaic acid treatment. FTY720 was used at 10μM and okadaic acid at 2.5nM. Data represented are mean of three independent experiments ± s.d.; * $P < 0.05$; ** $P < 0.01$.

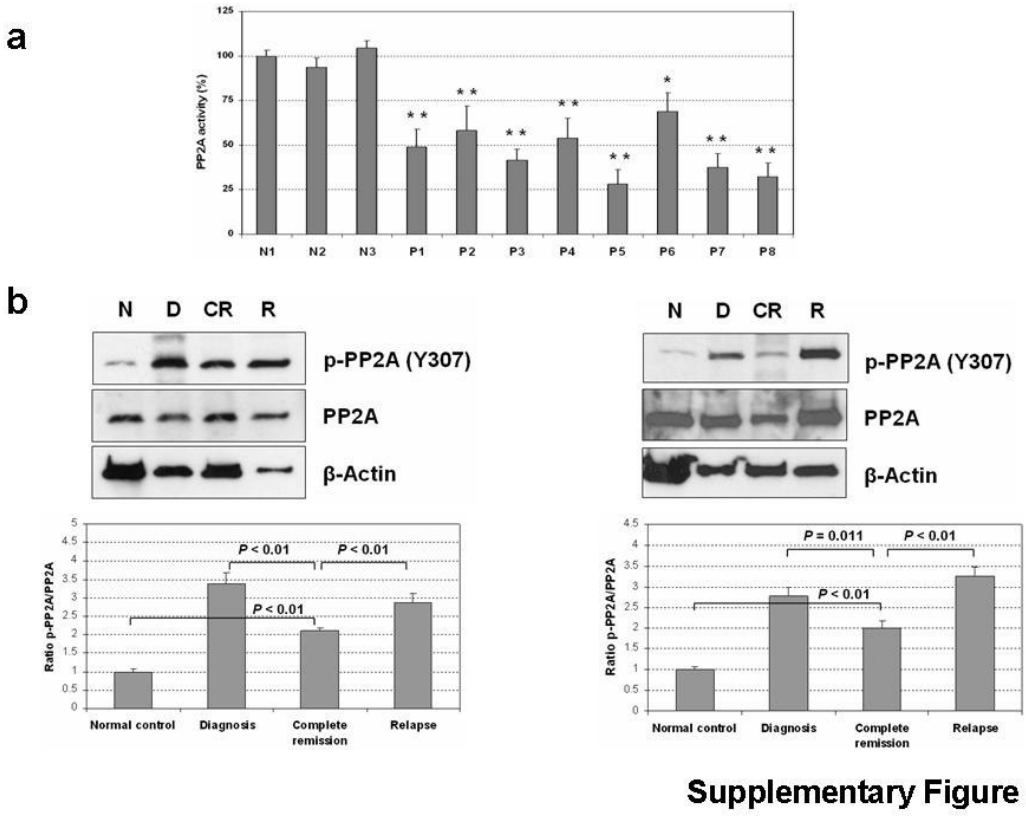


Supplementary Figure 2

Supplementary Figure 2 Forskolin and FTY720 treatments show a lesser effect on the proliferation of the PP2A low cell lines MUTZ-3 and Kasumi-1. FTY720 was used at 10µM, forskolin at 40µM and okadaic acid at 2.5nM. Data represented are mean of three independent experiments ± s.d.; * $P < 0.05$; ** $P < 0.01$.

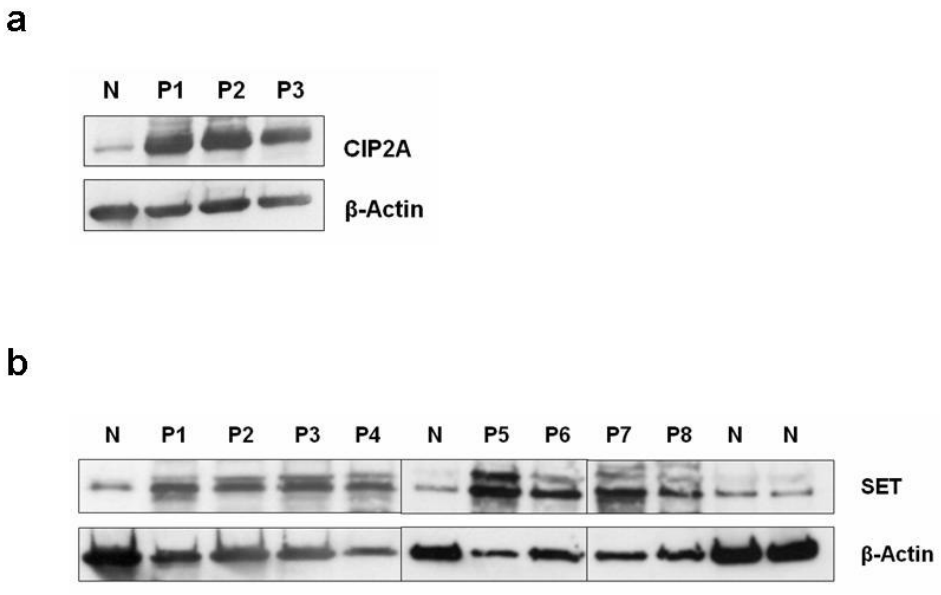


Supplementary Figure 3 Western blot and densitometric analysis showing PP2A activation status in two AML patients with samples at diagnosis, complete remission and relapse.



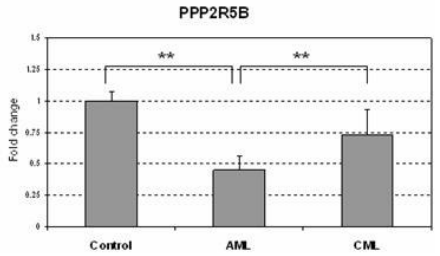
Supplementary Figure 4

Supplementary Figure 4 PP2A show reduced activity in AML patients. (**a**) PP2A activity in 8 AML patients at diagnosis compared to 3 normal controls; * $P < 0.05$; ** $P < 0.01$; N1-3: normal controls; P1-8: samples of AML patients at diagnosis (**b**) Comparison of PP2A activation in two AML patients with samples at diagnosis, complete remission and relapse, including densitometric analysis of p-PP2A/PP2A ratios; N: normal control; D: diagnosis; CR: complete remission; R: relapse.



Supplementary Figure 5

Supplementary Figure 5 Western blot analysis of CIP2A (**a**) and SET (**b**) expression in AML patient samples at diagnosis; N: normal control; P: patient sample.

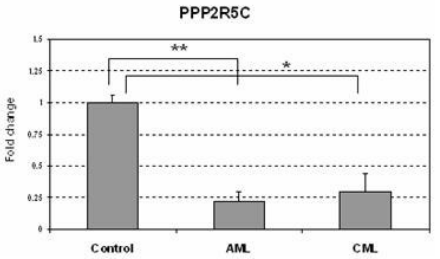


AML vs Control

Gene	Locus	Fold change	P-value	Adj. P-value	B
PPP2R5B	11q12-q13	0.451	<0.001	**0.001**	**1.534**
PPP2R5C	14q32	0.221	<0.001	**0.003**	**0.463**

CML vs Control

Gene	Locus	Fold change	P-value	Adj. P-value	B
PPP2R5B	11q12-q13	0.735	0.084	0.250	-5.557
PPP2R5C	14q32	0.296	0.002	**0.024**	-2.384



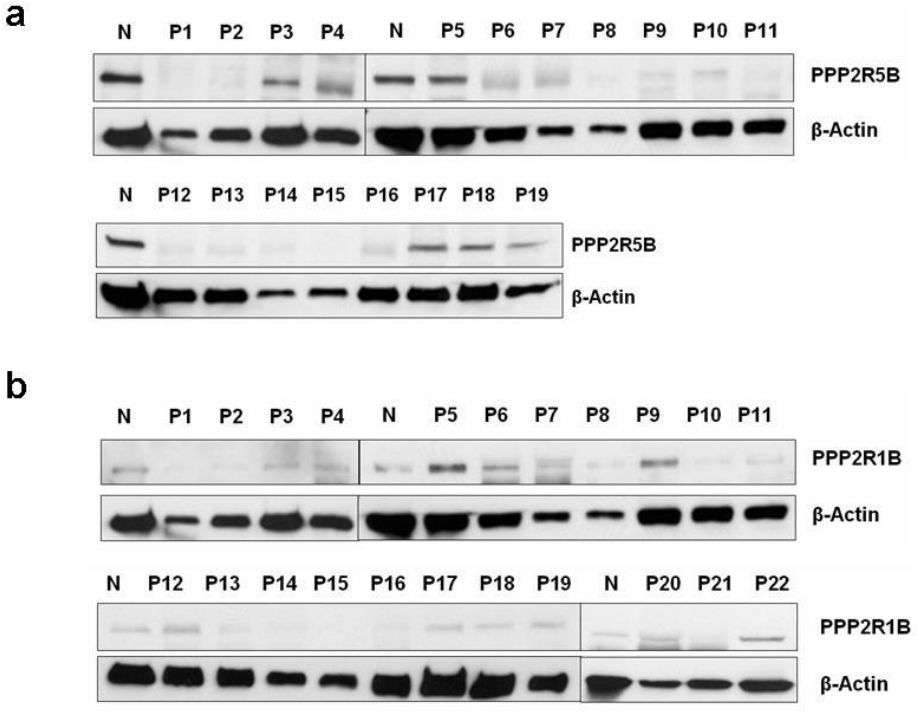
AML vs CML

Gene	Locus	Fold change	P-value	Adj. P-value	B
PPP2R5B	11q12-q13	0.618	<0.001	**0.019**	**1.342**
PPP2R5C	14q32	0.745	0.238	0.568	-5.493

*** Adj.P-value <0.05** **** B >0**

Supplementary Figure 6

Supplementary Figure 6 Significant alterations affecting PP2A subunits observed from analysis of expression array data. Samples were distributed in three different groups: “Control” including 3 normal control samples, “AML” including 12 AML cell lines, and “CML” including 4 CML cell lines. Data represented are the gene expression mean of each group ± s.d.



Supplementary Figure 7

Supplementary Figure 7 Western blot analysis of PPP2R5B (**a**) and PPP2R1B (**b**) expression in AML patient samples at diagnosis; N: normal control; P: patient sample.