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Estudio de la Función del Gen DOCK10, Inducible por IL-4 en Linfocitos B, como Factor de Intercambio de Nucleótidos de Guanina,y de los Perfiles de Expresión Génica y MicroRNA en Leucemias Linfocíticas Crónicas Tratadas con IL-4 *in vitro*

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La presentación de la Tesis Doctoral titulada "Estudio de la función del gen DOCK10, inducible por IL-4 en linfocitos B, como factor de intercambio de nucleótidos de guanina, y de los perfiles de expresión génica y microRNA en leucemias linfocíticas crónicas tratadas con IL-4 *in vitro*", realizada por Dña. Natalia Ruiz Lafuente, bajo mi inmediata dirección y supervisión cumpliendo todos los requerimientos para obtener el título de Doctor.

En Murcia, a 30 de Mayo de 2017

Antonio Parrado González

A mi **Padre**, desde arriba me cuidas, me acompañas, me alientas y me inspiras

---

"El futuro tiene muchos nombres. Para los débiles es lo inalcanzable. Para los temerosos, lo desconocido. Para los valientes es la oportunidad"

Victor Hugo

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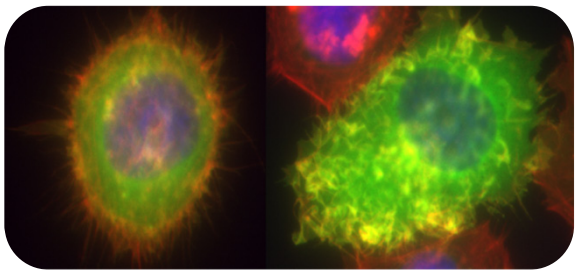

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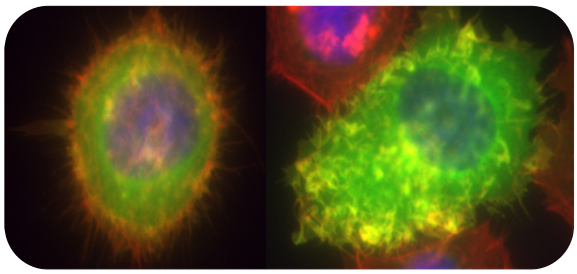
1. INTRODUCCIÓN



1.1 GTPASAS RHO

La familia de GTPasas Rho constituye un subgrupo dentro la superfamilia de pequeñas (~21 kDa) GTPasas Ras, proteínas monoméricas divididas en cinco sub-familias: Ras, Rho, Rab, Arf y Ran (Wennerberg y Der, 2004; Rossman et al., 2005). Se encuentran presentes en todos los eucariotas y controlan una amplia variedad de procesos celulares actuando como transductores de señales cuya función más conocida es la regulación del citoesqueleto de actina, siendo los miembros más estudiados RhoA, Rac1 y Cdc42.

Los miembros de la familia de GTPasas Rho están codificados por 23 genes en humanos. Se clasifican en 6 subfamilias según su homología en la secuencia primaria de aminoácidos, motivos estructurales y función biológica, mostrando sus miembros propiedades biológicas similares pero no idénticas (Figura 1) (Jaffe y Hall, 2005; Wennerberg y Der, 2004):

- **Subfamilia RhoA:** RhoA, RhoB y RhoC
- **Subfamilia Rac:** Rac1, Rac2, Rac3 y RhoG
- **Subfamilia Cdc42:** Cdc42,RhoQ/TC10, Rho J/TCL, RhoU/Wrch-1 y RhoV/Wrch-2/Chp
- Subfamilia Rnd: Rnd1, Rnd2 y RhoE/Rnd3
- Subfamilia RhoBTB: RhoBTB-1, RhoBTB-2 y RhoBTB-3
- Subfamilia RhoT: RhoT1/Miro-1 y RhoT2/Miro-2
- Y por último: **RhoD**, **RhoF/RIF** y RhoH/TTF que no pueden ser clasificadas de forma clara en ningún grupo de los anteriores.

Figura 1. **Árbol filogenético de la familia de GTPasas Rho y otros miembros representativos de la superfamilia Ras**. El análisis de la secuencia de aminoácidos de los dominios Rho mediante ClustalW, junto con datos funcionales muestra que la familia se divide en 6 subfamilias mayoritarias: RhoA, Rac, Cdc42, Rnd, RhoBTB y Miro (Wennerberg y Der, 2004).

Las GTPasas Rho, como la mayoría de los miembros de la superfamilia Ras, funcionan como "interruptores moleculares" por su capacidad de alternar entre dos estados, uno inactivo, unido a GDP, y otro activo, unido a GTP. Las GTPasas Rho se definen y distinguen de otras GTPasas por la presencia de un dominio GTPasa tipo Rho localizado entre la quinta cadena β y la cuarta hélice α en el dominio GTPasa (Valencia et al., 1991 y Wennerberg y Der, 2004). El dominio GTPasa muestra una identidad de aminoácidos del 30% con las proteínas Ras y un 40-95% dentro de la familia Rho y es común a todas las familias de GTPasas presentando una estructura secundaria que contiene seis láminas β y cinco hélices α. Es el responsable de la unión de GDP y GTP con alta afinidad y de su hidrólisis oscilando entre los dos estados mencionados anteriormente. Ambos estados de las GTPasas Rho difieren en su conformación primaria en dos regiones localizadas, switch I y II (Vetter y Wittinghofer, 2001). La conformación activa de la proteína, unida a GTP, aumenta su afinidad por proteínas efectoras aguas abajo. Cada proteína de la familia Rho reconoce múltiples efectores, y algunos efectores son reconocidos por varios miembros de la familia. La interacción y activación del efector estimula rutas de señalización que median en diversas funciones.

Las proteínas Rho se distinguen entre típicas o **clásicas**, las cuales son de pequeño tamaño (190-250 residuos) y presentan el dominio GTPasa y pequeñas extensiones en ambos extremos N y C-terminales. Sin embargo algunas de las **atípicas** contienen dominios adicionales bien definidos y pueden llegar a los 700 aminoácidos (Wennerberg y Der, 2004). En el grupo de las clásicas encontramos a las subfamilias Rho, Rac, Cdc42, RhoF y RhoD de manera que todas oscilan entre el estado activo unido a GTP e inactivo unido a GDP. La GTPasas atípicas las componen las subfamilias RhoBTB y Rnd,así como RhoU, RhoV y RhoH, las cuales están de forma permanente unidas a GTP por lo que se piensa que se regulan por otros mecanismos, como la fosforilación (Figura 2) (Heasman y Ridley, 2008).

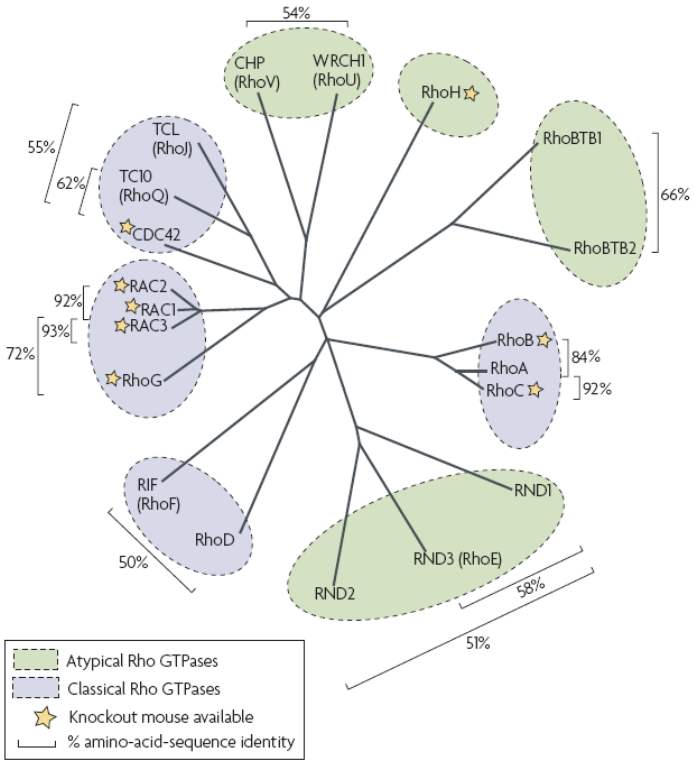


Figura 2. Árbol filogenético de la familia de GTPasas Rho basado en el alineamiento ClustlW de la secuencia de aminoácidos de 20 Rho GTPasas, en función del cual se incluyen en 8 subfamilias. La herramienta de alineamiento de pares de bases EMBOSS se usó para calcular los porcentajes de identidad de aminoácidos dentro de dichas familias. Además se distinguen en clásicas y atípicas según se explica en el texto (Heasman y Ridley, 2008).

1.1.1 Regulación de su actividad

- Regulación transcripcional, traduccional, modificaciones post-traduccionales y/o degradación vía proteosoma
- **Regulación del intercambio de nucleótidos**
- Regulación de la localización subcelular

Tengan expresión constitutiva o regulada transcripcional o traduccionalmente, en su mayoría, las GTPasas Rho regulan su actividad principalmente por la transición entre la forma unida a GDP y la unida a GTP. Esta conversión está controlada de forma bidireccional a través de dos tipos de proteínas reguladoras: **factores de intercambio de nucleótidos de guanina** (**GEFs,** del inglés, Guanine nucleotide exchange factors) y proteínas activadoras de GTPasas (**GAPs**, del inglés, GTPase activating proteins) (Figura 3).

Los **GEFs** estimulan la débil actividad intercambiadora intrínseca de las GTPasas Rho para promover el intercambio de GDP por GTP, dando lugar a su activación. Los GEFs se unen a la forma inactiva de la GTPasa, desestabilizando su interacción con el GDP y provocando su salida del complejo. La GTPasa está ahora en un estado de transición libre de nucleótidos que es estable gracias a la presencia del GEF. El GTP es mucho más abundante en el citoplasma que el GDP, por lo que su entrada al complejo está muy favorecida. La entrada de GTP provoca la disociación del GEF de la GTPasa, permitiendo su activación (Cherfils y Chardin, 1999; Schmidt y Hall, 2002). Por otro lado las proteínas **GAP** estimulan la actividad GTPasa intrínseca (que es muy baja de por sí) facilitando la conversión a la forma inactiva, unida a GDP.

Otra forma de regulación es por su correcta localización subcelular, que es fundamental para la asociación de las GTPasas Rho con sus efectores y, en algunos casos para la especificidad funcional de cada una. Por un lado sufren modificaciones post-traduccionales esenciales para su anclaje a membranas que consisten en la adición de lípidos isoprenoides a la secuencia llamada “caja CAAX” situada en el extremo C-terminal (donde C es Cys, A es cualquier aminoácido alifático y X es Met, Ser, Ala o Gln), además de la presencia de secuencias polibásicas ricas en lisina y arginina adyacentes a la caja CAAX (Wennerberg y Der, 2004). Por otro lado interaccionan con inhibidores Rho de disociación de GDP (**RhoGDIs**, del inglés, Rho GDP dissociation inhibitors), que retienen la forma unida a GDP en el citoplasma. La interacción del GDI con las GTPasas se produce a través de la región efectora lo que impide la unión tanto de GEFs como de GAPs como de efectores manteniéndolas en estado inactivo (Del Pozo et al., 2002; Hoffman et al., 2000; Scheffzek et al., 2000). Además los GDIs secuestran a

las GTPasas inactivas de las membranas a través de su unión al extremo C-terminal isoprenilado (Tybulewicz y Henderson, 2009).

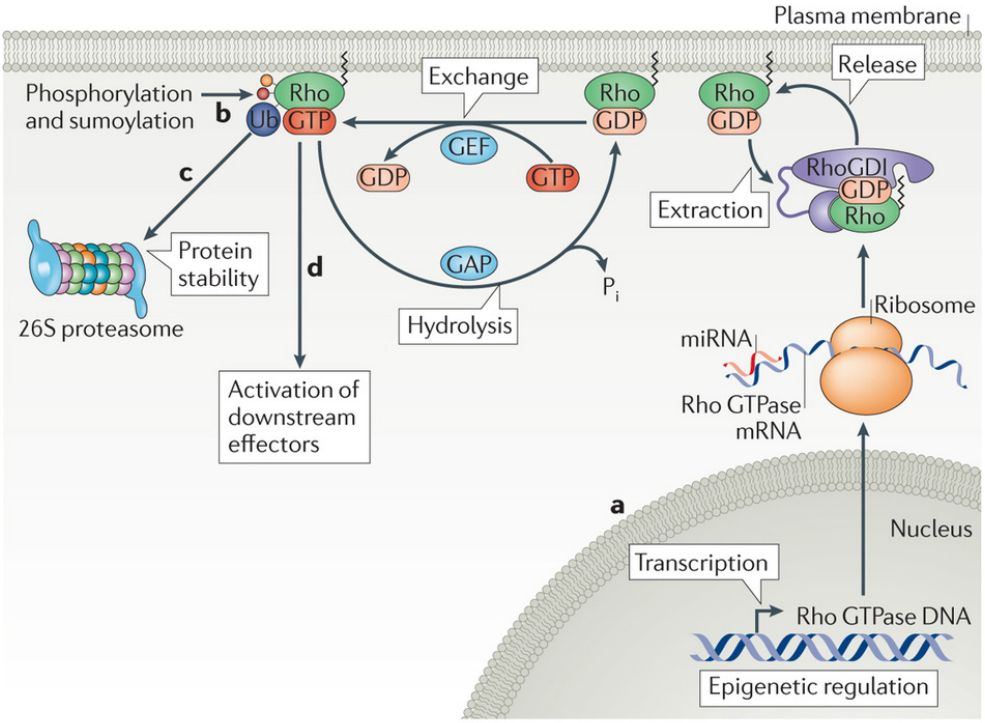


Figura 3. **Regulación de las GTPasas Rho**. Desde la expresión de la proteína hasta su activación por efectores hay un alto grado de regulación. GEFs, GAPs and RhoGDIs son las proteínas reguladoras clásicas del ciclo GTPasa. Los GEFs activan las GTPasas Rho catalizando el intercambio GDP por GTP, mientras que las proteínas GAPs estimulan la actividad GTPasa intrínseca inactivándolas. Los RhoGDIs extraen las GTPasas preniladas de la membrana uniéndose a los isoprenoides y secuestrándolas así en el citoplasma. Además se están poniendo de manifiesto otros mecanismos menos convencionales de regulación. (a) Control de la expresión de GTPasas Rho a nivel transcripcional epigenético y a nivel traduccional por microRNAs (miRNAs).(b) Modificaciones covalentes post-traduccionales de las GTPasas Rho tales como fosforilación y sumoilación que conducen a la activación o inactivación según el contexto celular. (c) Regulación del nivel de proteína a través del sistema ubiquitina-proteosoma. (d) la combinación de mecanismos reguladores clásicos y no convencionales asegura la apropiada activación espaciotemporal de las GTPasas Rho durante varios procesos celulares, incluyendo la regulación de la dinámica del citoesqueleto, polaridad celular y supervivencia (Hodge y Ridley, 2016).

1.1.2 Función biológica

El papel de las GTPasas Rho ha sido ampliamente estudiado, principalmente mediante estudios de sobreexpresión de GTPasas Rho dominantes negativos y constitutivamente activos en líneas celulares, que inhiben o sobre estimulan su ruta de señalización endógena, respectivamente (Heasman y Ridley, 2008). Se trata de mutaciones puntuales que generan formas constitutivamente activas o inactivas de estas proteínas. La sustitución G12V o Q61L da lugar a una forma constitutivamente activa de la GTPasa (Paterson et al, 1990; Ridley y Hall, 1992, Ridley et al., 1992) bloqueando la capacidad de hidrolizar el GTP, tanto intrínseca como inducida por GAPs (Diekmann et al., 1995; Garrett et al., 1989). Por otro lado, la sustitución T17N da lugar a un aumento de la afinidad de la GTPasa por GDP, lo que da lugar a una forma incapaz de interaccionar con efectores, y además se comporta como dominante negativo al bloquear la activación de las GTPasas endógenas al competir con ellas por los GEFs, a los que "secuestra" formando complejos inactivos (Feig, 1999; Ridley et al., 1992).

Como ya hemos dicho anteriormente, la GTPasa una vez activa se asocia transitoriamente con efectores aguas abajo, estimulando rutas de señalización que median en múltiples actividades celulares. Muestran funciones de estimulación del crecimiento y anti-apoptóticas así como de regulación de la expresión génica, a través de moléculas de señalización como NFκB, protein kinasas activadas por estrés y ciclina D1 (Pruitt y Der, 2001; Van Aelst y D'Souza-Schorey, 1997). Estimulan variedad de procesos incluyendo la reorganización del citoesqueleto de actina, morfogénesis, diferenciación, migración, división celular y adhesión (Wennerberg y Der, 2004; Rossman et al., 2005; Bishop y Hall, 2000; Heasman y Ridley, 2008; Tybulewicz y Henderson, 2009). Además diversas Rho GTPasas juegan un papel crucial en el desarrollo linfocitario (Tybulewicz y Henderson, 2009).La cantidad de efectores identificados para los miembros más representativos RhoA, Rac1, Cdc42 y otros, entre los que se incluyen serin/treonin quinasas, tirosin quinasas, lipasas, oxidasas y proteínas de sostén, refleja la complejidad y diversidad funcional de estas proteínas (Aspenström 1999; Bishop y Hall, 2000; Wennerberg y Der, 2004; Jaffe y Hall, 2005).

1.1.3 Papel en la reorganización del citoesqueleto de actina

Las GTPasas Rho son elementos clave en la regulación del citoesqueleto de actina afectando a procesos celulares tales como polaridad, migración, tráfico de vesículas y citocinesis. La mayoría de información de esta familia proviene de RhoA, Rac1 y Cdc42 que, como hemos dicho, son las más estudiadas. Las tres promueven la reorganización del citoesqueleto de actina, pero tienen distintos efectos sobre la forma y el movimiento celulares (Hall, 1998; Schmitz et al., 2000).

RhoA promueve la contractilidad actina-miosina y con ello la formación de fibras de estrés y adhesiones focales regulando la forma, adhesión y movilidad celulares. **Rac1** promueve la polimerización de actina y la formación de lamelipodios, que consisten en la protrusión de finas hojas de actina en el frente de avance o "leading edge" de células en migración. **Cdc42** produce formación de filopodios, que son finas extensiones digitiformes de citoplasma que contienen haces apretados de actina filamentosa y podrían estar implicados en el reconocimiento del entorno extracelular (Wennerberg y Der, 2004; Aspenström et al., 2004; Chhabra y Higgs, 2007; Heasman y Ridley, 2008). Al cultivar fibroblastos sobre un substrato plano sin estímulos de migración, éstos se adhieren y estiran para adoptar una forma alargada y se mueven al azar. La actividad de protrusión se determina por regulación local de la activación de GTPasas Rho. La regulación cruzada entre GTPasas favorece su coordinación. Así, Cdc42 contribuye a la actividad de Rac1 (Nobes y Hall, 1995; Yang et al., 2006), Cdc42 y RhoG contribuyen a la formación de lamelipodios a través de proteínas Rac (Monypenny et al., 2009), y Rac1 regula negativamente la formación de filopodios (Steffen et al., 2013). Estos efectos tan específicos sobre el citoesqueleto de actina son llevados a cabo a través de rutas de transducción de señales bien definidas controladas por cada GTPasa, que conducen a la formación (polimerización de actina) y organización/agrupación de los filamentos de actina (Figura 4).

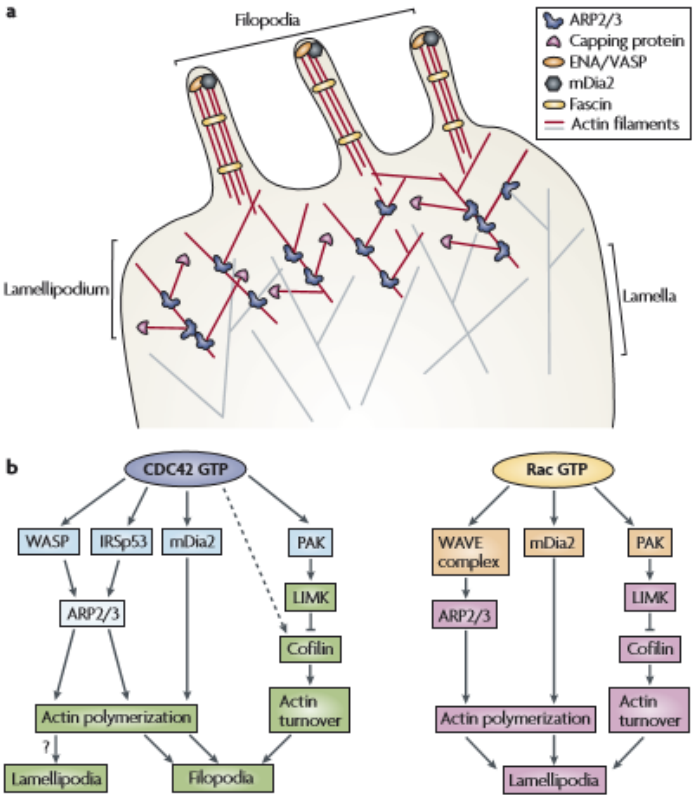


Figura 4. Formación de lamelipodios y filopodios a través de Rac y Cdc42. (a) En el "leading edge" o borde delantero de la célula existe una alta dinámica de formación de lamelipodios, los cuales se extienden gracias a la formación de nuevos filamentos de actina a partir de los ya existentes por activación del complejo Arp2/3. Esto conduce al ensamblaje de una red dendrítica de filamentos de actina ramificados. Las proteínas "capping" se unen a los extremos ramificados para terminar la elongación de los filamentos de actina. Rac activa la polimerización durante la formación del lamelipodio a través del complejo WAVE que activa Arp2/3, y posiblemente a mDia2, que liga filamentos de actina no ramificados por sus extremos. La lamela se localiza detrás del lamelipodio y se extiende atrás en el cuerpo celular. En la lamela, los filamentos de actina son más largos y menos ramificados, y la dinámica de actina se piensa que es independiente de la del lamelipodio. Los filopodios son finas protrusiones que contienen haces paralelos de filamentos de actina que se extienden desde el "leading edge" en la mayoría de células migratorias y probablemente funcionan como sondas sensoriales o para el establecimiento de contactos celulares. (b) Cdc42 induce la polimerización de actina a través de WASP o IRSp53 para inducir la formación de filamentos de actina ramificados mediante el complejo Arp2/3. Se desconoce si contribuye a la extensión del filopodio. Rac activa el complejo Arp2/3 a través del complejo WAVE. Cdc42 y Rac además inducen la polimerización de actina mediante la activación de mDia2. Rac y Cdc42 activan a la serina/treonina quinasa PAK que fosforila a LIMK, que a su vez fosforila e inhibe a cofilina, regulando así la renovación de los filamentos de actina (Heasman y Ridley, 2008).

1.2 PROTEÍNAS DOCK

Las proteínas DOCK (Dedicator of cytokinesis), también conocidas como proteínas CZH, son una familia compuesta por 11 miembros en mamíferos de GEFs para las pequeñas Rho

GTPasas (Figura 5) (Côté y Vuori, 2002; Meller et al., 2005; Fukui et al., 2001; Namekata et al., 2004; Miyamoto et al., 2007; Watabe-Uchida et al., 2006; Yelo et al., 2008; Meller et al., 2002). La familia DOCK está compuesta por proteínas de gran tamaño, en un rango comprendido entre 1.800 y 2.200 aminoácidos, y se definen por la presencia del dominio GEF conocido como CZH2. Las proteínas DOCK se agrupan en función de la homología de su secuencia en 4 subfamilias: A (DOCK180/DOCK1), B (DOCK4), C (Zir) y D (Zizimin). La subfamilia Zizimin se caracteriza por la presencia en su extremo amino-terminal de un dominio de homología con pleckstrina (PH) y está compuesta por 3 miembros, DOCK9, DOCK10 y DOCK11, también conocidos como Zizimin 1, Zizimin 3 y Zizimin 2, respectivamente (Meller et al., 2005; Yelo et al., 2008; Meller et al., 2002; Nishikimi et al., 2005; Lin et al., 2006).

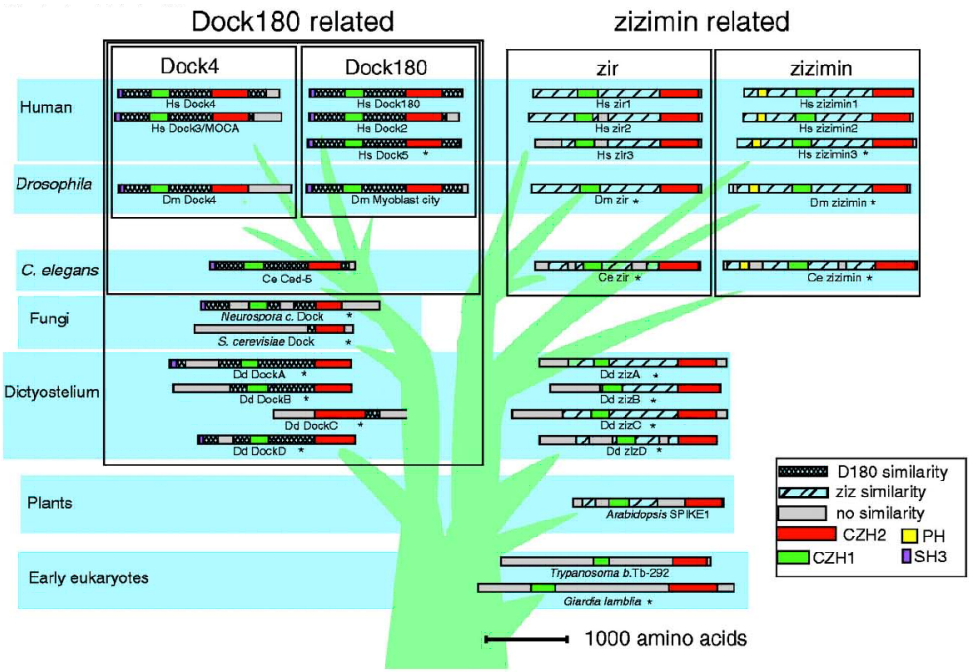


Figura 5. Estructura esquemática de las proteínas CZH. Se muestran los dominios CZH1 (CDM-zizimin homology 1), CZH2 (CDM- zizimin homology 2), SH3 (Src homology 3) y PH (pleckstrin homology). Marcadas con asteriscos aparecen las secuencias obtenidas mediante predicción computacional. El árbol representa su hipotética evolución basada en la similitud de su secuencia (Meller et al., 2005).

La proteína DOCK10 completa fue identificada y clonada por primera vez por nuestro grupo (Yelo et al., 2008), y demostramos la existencia de dos isoformas que designamos como DOCK10.1 (RefSeq No. NM_014689) y DOCK10.2 (RefSeq No. NM_001290263) (Alcaraz-García et al., 2011). Ambas isoformas fueron clonadas y secuenciadas quedando de manifiesto que se producen por "splicing", debido al uso alternativo de dos primeros exones mutuamente excluyentes y siendo idénticas a partir del segundo exón hasta el último (exón 56), difiriendo por tanto en su secuencia amino terminal y presentando un tamaño de 2.186 y 2.180 aminoácidos respectivamente (Figura 6). El estudio también puso de manifiesto que ambas isoformas se expresan en humano y en ratón, principalmente en linfocitos y órganos hematopoyéticos. Sin embargo DOCK10.1 se expresa principalmente en linfocitos T mientras que DOCK10.2 principalmente en linfocitos B normales (LBN) y malignos, como es el caso de la leucemia linfocítica crónica (LLC). El tratamiento con IL-4 induce un aumento en los niveles de ambas isoformas en LBN y LLC pero no en linfocitos T (Alcaraz-García et al., 2011). DOCK10 también se sobreexpresa en casos agresivos de carcinomas papilares de tiroides (Fluge et al., 2006), y en la transición epitelio-mesénquima de células de carcinomas escamosos (Humtsoe et al., 2012).

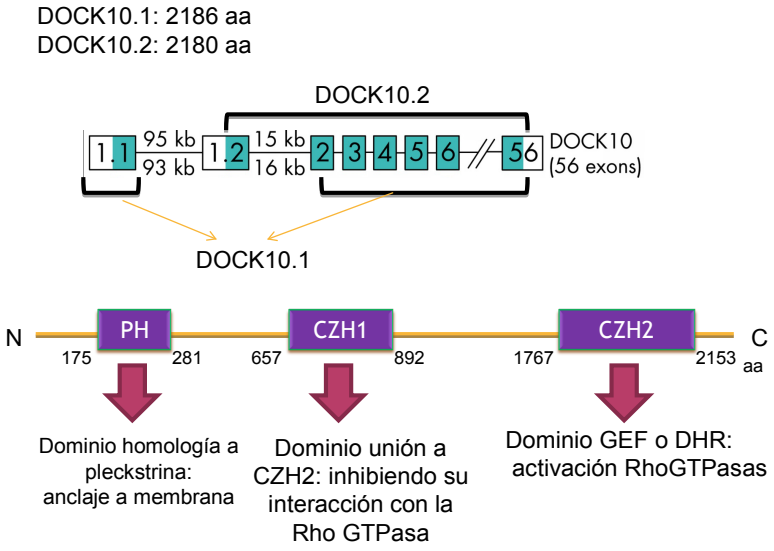


Figura 6. Esquema representativo del gen de DOCK10 que da lugar a dos isoformas y su secuencia de aminoácidos organizada en dominios.

En estudios previos se identifica a DOCK9 y DOCK11 como activadores específicos para la Rho GTPasa Cdc42 (Meller et al., 2002; Nishikimi et al., 2005; Lin et al., 2006; Meller et al., 2004; Meller et al., 2008; Kwofie y Skowronski, 2008; Yang et al., 2009; Kulkarni et al., 2011; Wu et al., 2011; Kuramoto et al., 2009). La literatura indica que DOCK10 podría actuar como GEF siendo activador de Rho GTPasas (Meller et al., 2005; Nishikimi et al., 2005; Gadea et al., 2008). Nishikimi et al. (2005) observaron una débil interacción del dominio CZH2 de Dock10 con Cdc42 y RhoJ, aunque la especificidad completa de DOCK10 no se conoce, pudiendo ser activador de Cdc42, como sus homólogos DOCK9 y DOCK11, o bien tener como dianas otras Rho GTPasas adicionales. Todo lo que sabemos sobre la función de DOCK10 proviene de un único estudio por silenciamiento mediante interferencia por miRNA, apareciendo DOCK10 como un factor que sustenta la morfología redondeada y el movimiento tipo ameboide en células de melanoma (Gadea et al., 2008).

1.3 INTERACCIONES FÍSICAS Y FUNCIONALES CON LAS GTPASAS RHO

La actividad GEF puede ser investigada en extractos celulares realizando los llamados ensayos de tipo “pulldown”. Las proteínas que se unen específicamente a la forma activada de laGTPasa Rho en cuestión se usan para precipitarla usando bolas o “beads” de sefarosa como soporte (Pellegrin y Mellor; 2008).

1.3.1 Ensayos GST pulldown

En nuestro caso, para poner de manifiesto las interacciones de DOCK10 con RhoGTPasas y su papel como activador de las mismas se realizaron ensayos *in vitro* del tipo Glutatión-S transferasa (GST) pulldown. Consisten en la precipitación de una proteína fusionada a GST a través de su purificación gracias a una matriz de afinidad en la que se haya fijado el tripéptido Glutatión (“beads” de Glutathione sepharose) (Figura 7).

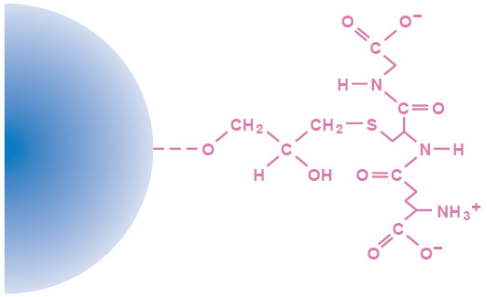


Figura 7. Estructura molecular del tripéptido glutatión unido a una matriz de sefarosa.

1.3.1.1 Ensayos de Interacción

Dichos ensayos consisten en la precipitación de la proteína GTPasa Rho, clonada en un vector de expresión junto al gen del enzima GST. La GST-GTPasa Rho es producida por transformación del vector en bacterias *E.coli*, obtención de lisados celulares y posterior purificación gracias a una matriz de afinidad en la que se haya fijado el tripéptido Glutatión ("beads" de "Glutathione sepharose"). Antes de la precipitación, la Rho GTPasa es incubada con el potencial GEF en condición libre de nucleótido o en presencia de GDP o GTP. Dichas condiciones tienen su sentido dado que los GEFs se diferencian de otras proteínas que interaccionan con las Rho GTPasas en su habilidad para unirse a ellas en su estado libre de nucleótido y no cuando están unidas a GDP o GTP (Meller et al., 2002; Cherfils y Chardin, 1999) (Figura 8). La condición de "beads" cargadas con la Rho GTPasa libre de nucleótido se establece suplementando los ensayos con ácido etilendiaminotetraacético (EDTA) que actúa quelando el magnesio, cofactor esencial para la unión del nucleótido. Sin embargo para la unión del nucleótido se suplementa con magnesio para mantener estable dicha unión. Si se produce la unión del GEF a la Rho GTPasa, al precipitar ésta, también precipitaremos el GEF y así podremos detectarlo mediante inmunoblotting.

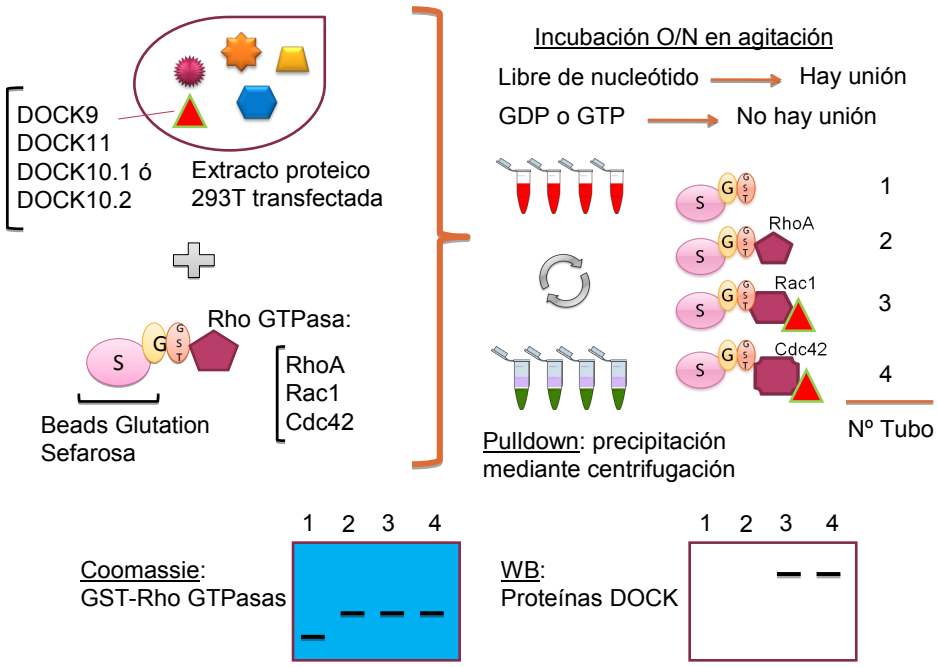


Figura 8. Esquema representativo de los ensayos de interacción "in vitro" del tipo Glutation-S transferasa (GST) pulldown.

1.3.1.2 Ensayos de Activación

Las GTPasasRho interaccionan con sus proteínas efectoras cuando están unidas a GTP (Aspenström et al., 1999). Rac1 y Cdc42 tienen como efectoras la familia de serin/treonin quinasas p21 activadas por Rac/Cdc42 (PAK). En la proteína efectora, al dominio que media en la interacción se le llama RBD (RhoGTPase-Binding Domain). Para Rac1 y Cdc42 la proteína efectora es PAK1 (p53 activated kinase) denominándose el dominio de interacción PAK-CRIB (Cdc42-Rac Interactive Binding Region), de unos 18 aminoácidos. La región PBD de PAK1 tiene una alta afinidad tanto para GTP-Rac1 como para GTP-Cdc42 y por tanto tiene un reducido ratio de hidrólisis intrínseco catalítico de Rac1 y Cdc42. Esto se aprovecha para realizar dichos ensayos de activación. Esta vez es el dominio CRIB de PAK1 el que se clona como proteína de fusión con GST en el vector pGEX-4-T-1 y se cargan "beads" de Glutation Sefarosa con ella (GST-PAK1). Se realizan los ensayos con estas beads de GST-PAK1 y lisados celulares de clones HeLa con expresión inducible de las proteínas DOCK. Al realizarse el "pulldown" de GST-PAK1, se habrá unido la GTPasa activa, cargada con GTP endógeno y el revelado será frente a la

GTPasa Rho que es activa en presencia de su GEF correspondiente. Gracias a estos ensayos se pone de manifiesto la especificidad de la GTPasa por su GEF.

1.4 LEUCEMIA LINFOCÍTICA CRÓNICA

La leucemia linfocítica crónica (LLC) es un tipo de síndrome linfoproliferativo crónico con expresión leucémica. Es una enfermedad maligna asociada a la edad avanzada, que cursa con la acumulación progresiva en sangre e infiltración en órganos hematopoyéticos de linfocitos B malignos CD5+CD23+. Éstos son maduros pero incapaces de realizar su función inmunitaria, con una escasa capacidad proliferativa, por lo que se postula que se acumulan a consecuencia de una inhibición de la diferenciación y la apoptosis. En la LLC, los linfocitos B malignos infiltran progresivamente la médula ósea, los tejidos linfáticos (ganglios y bazo fundamentalmente) y otros órganos como por ejemplo el hígado (Kipps et al., 2017).

La LLC es la leucemia más frecuente en los países occidentales (20-40% del total de leucemias) siendo una enfermedad rara en países orientales. En España cada año se diagnostican alrededor de 30 nuevos casos por millón de habitantes. Habitualmente, la LLC afecta a personas mayores de 60 años (edad media 70 años; tan sólo el 20% son menores de 65 años), siendo extremadamente excepcional en niños, y progresa muy lentamente. En muchos casos las personas que padecen esta enfermedad no presentan síntomas durante años.

1.4.1 Evolución clínica. Síntomas

La evolución clínica es heterogénea. Aproximadamente la mitad de los pacientes viven durante décadas sin requerir tratamiento alguno, mostrándose asintomáticos y siendo diagnosticados al detectarse una linfocitosis en una analítica de sangre rutinaria. Sin embargo, la otra mitad pueden empezar a mostrar síntomas o progresan a estadios tardíos de la enfermedad requiriendo tratamiento. Los síntomas habituales incluyen fatiga, pérdida de peso, sudor nocturno excesivo, plenitud abdominal con saciedad temprana y mayor frecuencia de infecciones, que pueden estar asociadas con hipogammaglobulinemia. Además también pueden presentar agrandamiento de los ganglios linfáticos (que pueden ser fácilmente palpables en tres regiones: cervical, axilar y inguinal-femoral), hepatomegalia y esplenomegalia (Kipps et al., 2017).

1.4.2 Tratamiento

El tratamiento de los pacientes con LLC puede incluir quimioterapia, combinación de quimioterapia e inmunoterapia, o agentes cuya diana sea rutas de señalización que promueven el crecimiento y/o la supervivencia de las células LLC, por ejemplo señalización BCR y BCL-2 (Eichhorst et al., 2015). **Quimioterapia**: se utilizan agentes quimioterápicos como análogos de la purina, siendo el más común fludarabina (pero también pentostatina o cladribina) combinados con agentes alquilantes como clorambucilo, ciclofosfamida o bendamustina. **Quimioinmunoterapia**: se utilizan anticuerpos monoclonales anti-CD20 como rituximab, obinutuzumab u ofatumumab en combinación con quimioterapia**. Inhibidores de la señalizacion BCR**: se utilizan 3 tipos principales de drogas que pueden inhibir la ruta de señalizacion por BCR en pacientes LLC (inhibidores de BTK, inhibidores de PI3K e inhibidores de SYK (tirosin quinasa de bazo)) (de Rooij et al., 2012, Ponader et al., 2012). **Inhibidores de BCL-2**: se utiliza Venetoclax que es una pequeña molécula que mimetiza a BH3 inhibiendo a BCL-2 (Souers et al., 2013). Esta droga es muy potente induciendo apoptosis en células LLC, posiblemente disminuyendo la capacidad de BCL-2 para secuestrar la molecula pro-apoptótica BCL2L11 (Del Gaizo Moore et al., 2007).

1.4.3 Clasificación

Se utilizan dos sistemas ampliamente extendidos de clasificación clínica para dividir a los pacientes con LLC en tres grandes grupos pronóstico, en función de su evolución y gravedad (Tablas 1 y 2). El sistema de Rai es comúnmente usado en Estados Unidos, mientras que el de Binet es más usado en Europa (Kipps et al., 2017).

Tabla 1. Sistema de clasificación de Rai

Grupo de riesgo	Características Clínicas	Esperanza de Vida media
Bajo riesgo (Estadio Rai 0/I)	Linfocitosis sin citopenia, linfoadenopatía o esplenomegalia	13 años
Riesgo Intermedio (Estadio Rai II)	Linfocitosis, linfoadenopatía y/o esplenomegalia, pero sin citopenia	8 años
Alto Riesgo (Estadio Rai III/IV)	Linfocitosis y citopenia (nivel de hemoglobina ≤11 g/dl y/o recuento plaquetario ≤100,000 células/µl)	2 años

Tabla 2. Sistema de clasificación de Binet

Grupo de riesgo	Características Clínicas	Esperanza de Vida media
Bajo riesgo (Estadio Binet A)	Menos de tres sitios* palpablemente agrandados sin citopenia	13 años
Riesgo Intermedio (Estadio Binet B)	Tres o más sitios* palpablemente agrandados sin citopenia	8 años
Alto Riesgo (Estadio Binet C)	Citopenia (nivel de hemoglobina ≤10 g/dl y/o recuento plaquetario ≤100,000 células/µl)	2 años

* Hay 5 sitios en órganos linfoides: nódulos cervicales, axilares e inguinales, bazo e hígado.

1.4.4 Etiología

La base biológica de esta enfermedad es compleja y los cambios moleculares que conducen a su patogénesis son aún poco conocidos. No se considera una enfermedad hereditaria aun cuando existen antecedentes familiares en algunos casos. No hay una causalidad comprobada con la historia clínica del paciente (otras enfermedades o circunstancias del enfermo) y no está establecida una relación directa con radiaciones, tóxicos u otras sustancias externas. Inciden aspectos inmunológicos (estimulación por determinados antígenos o infecciones), influencia del micro medioambiente, evolución natural de las poblaciones de linfocitos B envejecidas, etc.

1.4.4.1 Alteraciones genéticas

Se incluyen alteraciones cromosómicas, mutaciones, alteraciones en la expresión de microRNAs (miRNAs) y modificaciones epigenéticas.

- Alteraciones cromosómicas

Aproximadamente el 80% de los pacientes presentan al menos una de las cuatro alteraciones cromosómicas más comunes: deleción 13q (del(13q)), del(11q), del(17p) y trisomía 12 (Dohner et al., 2000). Del(13q) es la más común, encontrándose en más del 50% de los pacientes y está asociada a mejor pronóstico. Del(17p) aparece en el 7% de los pacientes y está asociada a la pérdida del gen supresor de tumores TP53 (40), mientras que del(11q) aparece en el 18% de los pacientes y se suele asociar con alteraciones en el gen ATM (“ataxia telangiectasia mutated”) que codifica una proteína implicada en la reparación de DNA. Del(11q) y del(17p) se consideran de mal pronóstico (Dohner et al., 2000). La trisomía 12 se encuentra en el 16% de los pacientes asociándose a un pronóstico intermedio.

- Mutaciones somáticas

Se han producido grandes avances en los últimos años gracias al proyecto mundial para la secuenciación del genoma del cáncer gestionado por el Consorcio Internacional del Genoma del Cáncer (ICGC) dentro del cual se encuentra el Proyecto Genoma de la LLC (2009 - 2012) de cuya realización se encargó España. El objetivo fue secuenciar el genoma completo de los pacientes y poder establecer las homogeneidades y variabilidades para poder hacer un enfoque de diagnóstico más personalizado y eficaz. En 2011 se publicaron, en la revista Nature, los datos obtenidos de la secuenciación completa del genoma de 4 pacientes con LLC identificándose 46 mutaciones somáticas que potencialmente podrían estar afectando la función génica. Dichas mutaciones, a su vez, se analizaron en 363 pacientes con LLC identificando 4 genes mutados de forma recurrente: notch 1 (NOTCH1), exportina 1 (XPO1), "myeloid differentiation primary response gene 88" (MYD88) y "kelch-like 6" (KLHL6). Estos patrones reiterados de mutaciones somáticas, apoyados por análisis clínicos y funcionales, suponen cambios oncogénicos que contribuyen a la evolución clínica de la enfermedad. Este estudio ha supuesto el primer análisis exhaustivo en la LLC combinando secuenciación de genoma completo con información clínica. La información contenida en él podrá ser útil para mejorar el diagnóstico y caracterización de los pacientes y promover nuevos enfoques terapéuticos (Puente et al., 2011). Más tarde, en 2015, se amplió el número de pacientes a 506 y se identificaron nuevas mutaciones recurrentes en regiones no codificantes (Puente et al., 2015).

Gracias a estos datos recientemente aportados por la secuenciación masiva y del exoma, se ha demostrado que la LLC presenta un alto grado de variabilidad genética (Fabbri G et al., 2011; Pleasance et al., 2010; Puente et al., 2011; Puente et al., 2015; Wang et al., 2011). La detección de mutaciones somáticas y su frecuencia es variable, lo que quizás refleja diferencias en la composición de las cohortes estudiadas. Sin embargo estudiando grupos cada vez más amplios y diversos se podrían descubrir nuevos genes alterados candidatos a ser responsables de la LLC.

Se han encontrado mutaciones somáticas recurrentes en genes implicados en diversas funciones: daño del DNA (TP53 y ATM), procesamiento del RNA mensajero (mRNA) (SF3B1 y XPO1), modificación de la cromatina (HIST1H1E, CHD2 y ZMYM3), ruta de señalización WNT, ruta de señalización Notch (NOTCH1) y rutas inflamatorias (MYD88). Otras mutaciones, tales como las encontradas en EGR2 o BRAF, pueden afectar a la señalización y transcripción de la célula B (Damm et al., 2014). Investigaciones posteriores apoyan el papel funcional de estas alteraciones, como es el caso de experimentos de silenciamiento de genes de la ruta WNT en

células LLC primarias cuya consecuencia fue una disminución en la viabilidad celular (Wang L et al., 2014). Mutaciones en POT1, implicado en la protección de los telómeros, impiden la unión de esta proteína a su lugar en el telómero, induciendo de un desarrollo anormal de los telómeros y aberraciones cromosómicas. Las mutaciones en SF3B1 se asocian a "splicing" aberrante del mRNA (Wang L et al., 2014; Ferreira et al., 2014; Quesada et al., 2012) y alteración de la respuesta al DNA dañado (Te Raa et al., 2015).

- Alteraciones en miRNA

La LLC fue la primera enfermedad humana asociada con alteración en miRNAs. MiR-15a y miR-16-1 (Calin et al., 2002) están delecionados, alterados o infra-expresados en aproximadamente el 60% de los pacientes con LLC (Calin et al., 2002). Ambos, miR-15a y miR-16-1 tienen como diana a BCL2 y MCL1 (Cimmino et al., 2005) que codifican proteínas apoptóticas de la familia BCL-2 (Fabbri M et al., 2011). La reducción de la expresión o pérdida de estos miRNAs estaría potenciando la expresión de sus genes diana.

- Cambios epigenéticos

El programa epigenético celular tiene una importante función en el desarrollo de la LLC. El epigenoma en la LLC muestra una hipometilación global combinado con una hipermetilación local, como se observa en otros cánceres (Cahill et al., 2013; Wahlfors et al., 1992; Ziller et al., 2013). Aunque los patrones de metilación han resultado ser muy heterogéneos (Puente et al., 2015; Kulis et al., 2012; Landau et al., 2014; Oakes et al., 2014; Pei et al., 2012; Queiros et al., 2015), la firma molecular de metilación clasifica distintos grupos clínicos en la LLC (Kulis et al., 2012; Bhoi et al., 2016). Así por ejemplo las células LLC con o sin mutación en IGHV muestran distintos patrones de metilación.

1.4.5 Factores o marcadores pronóstico. Perfiles moleculares

Los factores y/o marcadores pronóstico pueden ayudar a identificar pacientes que requieran terapia temprana e incluyen características clínicas, genéticas, y moleculares. Entre los factores asociados a mal pronóstico se incluyen el sexo masculino, edad ≥65 años, o un mal estado general debido a las comorbilidades.

Como hemos dicho, más del 80% de LLC presentan una o más anomalías citogenéticas (del(13q), del(11q), del(17p), +12, etc) con distinto valor pronóstico. Los pacientes con LLC se pueden clasificar en dos grupos pronósticos, uno con una evolución más indolente, asociado a

la presencia de mutaciones en la región variable del receptor de células B o cadena pesada variable de la inmunoglobulina (IgVH) y otro con evolución más agresiva, asociado a la ausencia de mutaciones en IgVH. También son marcadores de peor pronóstico en LLC el alto nivel de expresión de ZAP70, CD38 y CD49d/ITGA4 (Schena el al., 1992; Hanada el al., 1993; Pepper et al., 1997; Chiorazzi, 2012) , presencia de del(17p)o del(11q), altos niveles en suero de β2-microglobulin (>3.5 mg/l) (Hallek et al., 1996), cariotipo complejo (presencia de tres o más aberraciones cromosómicas en un test de cariotipo) (Le Bris et al., 2016; Thompson et al., 2015), o un alto recuento linfocitario (>50,000 cel/µl) y/o situación de etapa tardía de la enfermedad en la presentación inicial. Del(17p) se suele asociar con mutaciones que inactivan TP53, siendo marcador de mal pronóstico y respuesta al tratamiento con quimioterapia convencional (Byrd et al., 2006).

Parece probable que las LLC, sean de un grupo pronóstico u otro, se originen de la transformación oncogénica de un precursor linfocitario B que ha sido estimulado antigénicamente (Gribben, 2010). A pesar de su heterogeneidad, los análisis de expresión génica mediante microarrays han definido un perfil homogéneo para las LLC que se conoce como firma molecular de esta patología, si bien existen algunos genes y miRNAs cuyos perfiles de expresión génica (PEG) son específicos en los distintos grupos pronósticos (Klein et al., 2001; Rosenwald et al., 2001; Zheng et al., 2002; Jelinek et al., 2003; Dürig et al., 2003; Fält et al., 2005; Hüttmann et al., 2006; Joshi et al., 2007; Rodríguez et al., 2007; Fulci et al., 2007; Del Giudice et al., 2009; Porpaczy et al., 2009; Friedman DR et al., 2009; Kanduri et al., 2010). Como hemos dicho los PEG en LLC son relativamente homogéneos, considerando que existen características específicas de LLC que claramente discriminan las células LLC de las células B de otras patologías relacionadas y de las células B normales (Klein et al., 2001; Rosenwald et al., 2001; Stratowa et al., 2001; Zheng et al., 2002; Jelinek et al., 2003; Wang et al., 2004; Dürig et al., 2003), mientras que las características específicas para los grupos pronósticos de LLC se basan en diferencias más sutiles (Wiestner et al., 2003; Stankovic et al., 2004; Rodríguez et al., 2007; Seifert et al., 2012). Las células LLC mueren espontánea y rápidamente *in vitro* debido a la falta de señales esenciales proporcionadas por el micro medio ambiente natural (Douglas et al., 1997). Las células LLC interactúan con células estromales de la médula ósea y con células T, células presentadoras de antígeno y células dendríticas dentro de los centros de proliferación de los nódulos linfáticos (o pseudofolículos). Citocinas, quimiocinas, integrinas y otros ligandos y receptores juegan un papel en la proliferación y supervivencia dentro de estos nichos celulares (Burger y Montserrat, 2013). Algunos estudios señalan una posible activación de la señalización por IL-4 en la LLC, y en ensayos clínicos, el tratamiento con IL-4 ha resultado perjudicial en esta patología (Lundin et al., 2001).

1.5 INTERLEUCINA-4

La Interleucina-4 (IL-4) es una citocina secretada por células T activadas, células NK-T, basófilos, eosinófilos y mastocitos. La estimulación paracrina a través del receptor de membrana de IL-4 (IL-4R) induce la cascada de señalización que conduce a la maduración de los precursores de células B en células secretoras de inmunoglobulinas y células presentadoras de antígeno, proliferación de células B activadas e inducción de cambio de isotipo a IgE (Okada et al., 2003). Como se muestra en la Figura 9, IL-4R activado fosforila a JAK1 y JAK3. JAK1 fosforila a STAT6, el cual homodimeriza y entra al núcleo para regular la expresión génica. JAK1 y JAK3 conducen señales anti-apoptóticas a través de las rutas mitocondrial y de PI3K/AKT y a través de la ruta Ras/MAPK y la activación de NFκB (Zamorano et al., 2001). La activación de NFκB es anti-apoptótica en LLC (Furman et al., 2000; Cuní et al., 2004). En células B, la IL-4 induce preferentemente la ruta no-canónica de NFκB (Thieu et al., 2007). La IL-4 induce eficientemente la fosforilación y activación de STAT6 en LLC (Bhattacharya et al., 2015). Sin embargo, la unión de NFκB al promotor de IGHE, CD86 y MHCII es necesariapara la unión de STAT6 y la transcripción (Thieu et al., 2007; Shen y Stavnezer, 1998; Messner et al., 1997).

La IL-4 protege eficientemente a las células LLC de la apoptosis espontánea o de la muerte por agentes como fludarabina y clorambucil (Douglas et al., 1997; Dancescu et al., 1992; Steele et al., 2010). Se ha comprobado que las células LLC son más propensas a la apoptosis espontánea que las células B normales (CBN) (Douglas et al., 1997) y aquellas que expresan marcadores de buen pronóstico también lo son más que las de mal pronóstico (Coscia et al., 2011). La IL-4 actúa de manera paracrina más que autocrina en LLC (Mainou-Fowler et al., 2001). Los PEG en linfoma folicular sugieren que una conexión dependiente de IL-4 entre células T y células B malignas sustenta la tumorigénesis (Pangault el al., 2010). Igualmente, La IL-4 podría jugar un papel en la patogénesis y progresión de la LLC. Varios estudios se han centrado en identificar las dianas de la IL-4 en esplenocitos B de ratón (Schroder et al., 2002), algunos subtipos de linfoma (Lu et al., 2005), y otros tipos celulares no B. Sin embargo, la expresión génica en respuesta a la IL-4 en LLC es poco conocida. A través de su efecto citoprotector, la ruta de la IL-4 podría sostener la evasión de la apoptosis de las células LLC, y de este modo contribuir a la leucemogénesis.

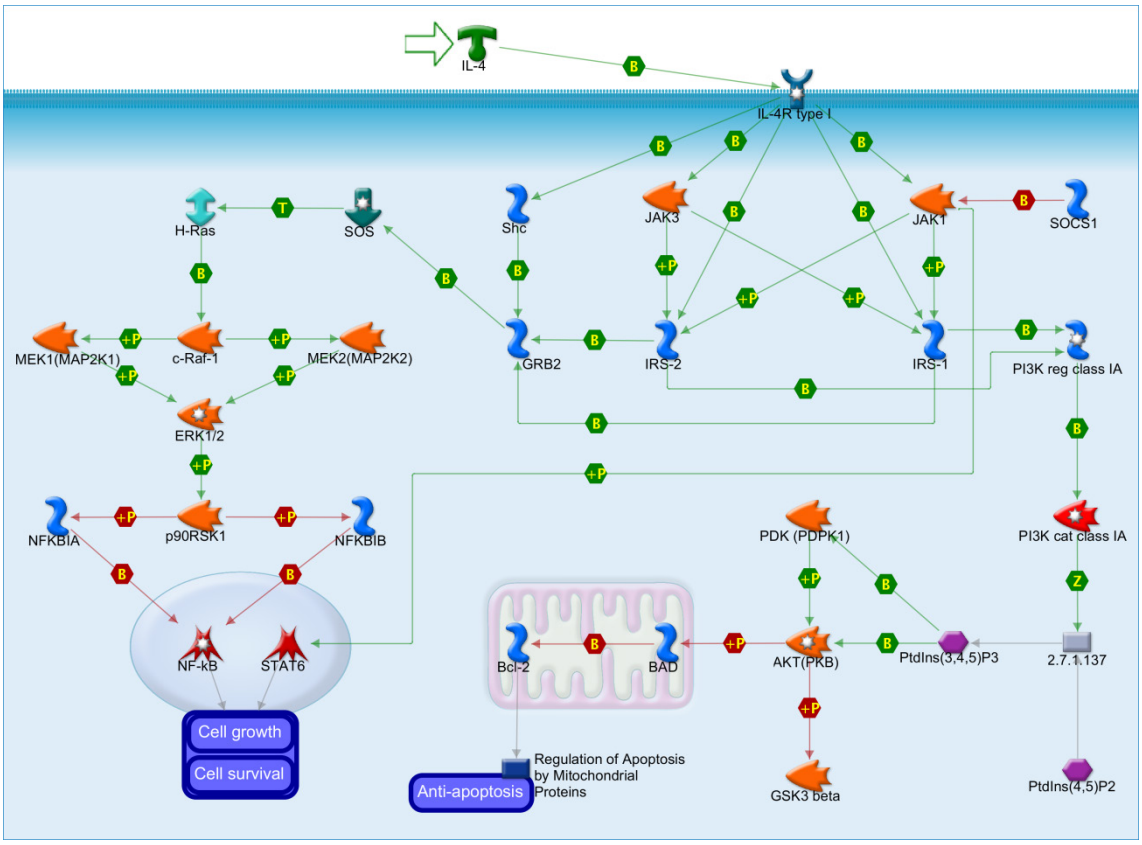


Figura 9. Mapa de la ruta "Immune response_IL-4-antiapoptotic action" (MetaCore, Thompson Reuters, https://portal.genego.com).

1.6 MIRNAs

1.6.1 Definición

Los miRNAs son pequeños RNAs no codificantes (ncRNA) que típicamente inhiben la traducción y estabilidad delosmRNAs, controlando genes implicados en procesos celulares como inflamación, regulación del ciclo celular, respuesta a estrés, diferenciación, apoptosis y migración. Por tanto, los miRNAs están implicados en la regulación de prácticamente todas las rutas de señalización dentro de una célula y se ha demostrado que su desregulación desempeña un papel esencial en el desarrollo y progresión del cáncer (Di Leva et al., 2014).

Los primeros miRNAs fueron descubiertos en 1993, cuando se describió por primera vez, en el nematodo *Caenorhabditis elegans*, la naturaleza de un gen denominado Lin-4 como un pequeño ncRNA, con complementariedad hacia el gen Lin-14, capaz de disminuir la expresión de la proteína LIN-14159 (Lee et al., 1993). En aquel momento se consideró que se trataba de un mecanismo único, siendo este ncRNA una herramienta específica empleada por

estos nematodos para controlar de alguna manera la expresión génica de genes codificantes heterocrónicos (Di Leva et al., 2014). Años después, Reinhart et al. (2000) identificó otro gen heterocrónico en *C. elegans*, Let-7, que también se trataba de un pequeño ncRNA, que al igual que Lin-4, era capaz a través de interacción RNA-RNA con la región 3'-UTR (del inglés, untraslated región) de los genes diana, comenzar la cascada temporal de genes heterocrónicos reguladores, reprimiendo Let-7 la expresión de Lin-41, Lin-14, Lin-28, Lin42, y Daf-12 durante el desarrollo de *C. elegans* (Reinhart et al., 2000). Este descubrimiento suscitó un gran interés y en 2001 diferentes grupos identificaron otros pequeños ncRNAs en varias especies de vertebrados incluyendo humanos, demostrándose la existencia de un gran grupo de estos pequeños ncRNAs (posteriormente denominados miRNAs) con una potencial función reguladora (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee et al., 2001). Desde entonces se ha demostrado que están conservados en la evolución siendo expresados de forma ubicua en todos los organismos eucariotas. Actualmente en la base de datos miRBase (última versión en Junio de 2016) figuran 2661 miRNAs maduros humanos.

La mayoría de genes miRNA están localizados en regiones bastante distantes de los otros genes anotados sugiriendo que representan unidades transcripcionales independientes. Alrededor de la mitad de los genes miRNA se encuentra formando parte de familias (miRNAs que comparten la secuencia que se une a la región complementaria del mRNA diana, conocida como región semilla) o están agrupados en "clusters" formados por miRNAs (generalmente sus isoformas o parálogos) localizados en la misma región dentro del genoma (Kim, et al., 2009). Al compartir la región semilla, los miembros de una misma familia de miRNAs son potenciales reguladores del mismo conjunto de genes y normalmente se transcriben multicistrónicamente. Por ejemplo el cluster miR-17~92 produce un único precursor policistrónico que es madurado en 6 miRNAs diferentes que se clasifican en 4 familias: miR-17 (miR-17 and 20), miR-18, miR-19 (miR-19a y 19b), y miR-92 (Tanzer, 2004). Una proporción minoritaria de miRNAs son intragénicos, ya que se localizan dentro de otro gen pudiendo ser el gen huésped codificante o no. La mayor parte de éstos se localizan en regiones intrónicas, aunque también pueden estar ubicados dentro de regiones exónicas.

1.6.2 Biogénesis

Los miRNAs se definen como ncRNAs de cadena sencilla de entre 19-25 nucleótidos de longitud, generados a partir de transcritos endógenos en horquilla (Bartel, 2004). Regulan de forma negativa la expresión génica uniéndose por complementariedad de bases parcial a sitios

de los mRNA diana, generalmente en el extremo 3'-UTR reprimiendo su traducción. Se generan en un proceso multietapa tal y como se describe en la figura 10.

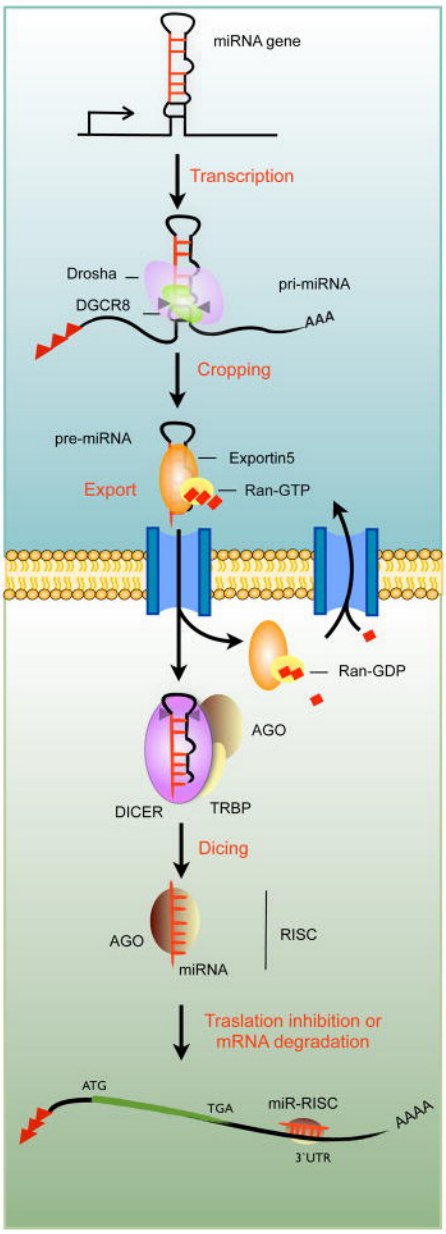


Figura 10. Biogénesis de miRNAs, un proceso en varias etapas: transcripción, corte en el núcleo (nuclear cropping), transporte al citoplasma y corte en el citoplasma (cytoplasmic dicing). Los genes miRNAs se transcriben generalmente en transcritos de RNA con caperuza en 5' y cola de poli-adeninas en 3' por la RNA polimerasa II. Su longitud supera al tamaño final del miRNA maduro y contiene regiones complementarias lo que permite su apareamiento. Esto conlleva que se forman regiones horquilla. Este RNA bicatenario primario largo es conocido como pri-miRNA. Posteriormente el complejo proteico llamado Microprocesador (formado por la nucleasa Drosha17 y la proteína de unión a RNA de doble hélice Pasha) realiza un primer procesamiento en el núcleo, cortando bases de la horquilla, formando estructuras más cortas en forma de tallo-lazo (stem-loop) conocidas como pre-miRNA. Este pre-miRNA es transportado desde el núcleo al citoplasma por el transportador Exportina 5 (XPO5)/RanGTP. Una vez en el citoplasma, la endonucleasa Dicer corta el pre-miRNA, generando un dúplex (dos moléculas complementarias cortas), que es incorporado al complejo de silenciamiento inducido de RNA (RISC). Una de las hebras es retenida por este complejo, la cual constituye el miRNA maduro que se une al mRNA diana causando la inhibición de su traducción, mientras que la otra es degradada (Di Leva et al., 2014).

1.6.3 Mecanismo de acción y funciones

El principal mecanismo de regulación por miRNAs está mediado por su capacidad de unión (por homología parcial) a secuencias complementarias, generalmente en la región 3' UTR, de los mRNA diana, provocando silenciamiento génico a nivel postranscripcional,

pudiendo suprimir la traducción o degradando la molécula de mRNA. De hecho, existen trabajos publicados que indican que los miRNA de células animales reprimen la expresión génica de cuatro formas diferentes: degradación de la proteína durante la traducción, inhibición de la elongación de la traducción, terminación prematura de la traducción (disgregación de los ribosomas), e inhibición de la iniciación de la traducción. Desentrañar esta complejidad mecanística ha sido y es objeto de intensa experimentación (Eulalio et al., 2008). Incluso se ha descrito que la acción de los miRNAs puede asociarse al aumento de la expresión de genes diana, indicando que los mecanismos por los cuales actúan los miRNAs son aún más complicados (Vasudevan et al., 2007).

La mayoría de miRNAs no son complementarios a un solo mRNA específico, sino que regulan simultáneamente más de un gen, de hecho, un único miRNA puede reprimir cientos de mRNAs (Hayden, 2008). Por otro lado, un mRNA puede estar regulado por diferentes miRNA ejerciendo incluso funciones biológicas similares, amplificando de esta manera sus acciones (Bueno et al., 2008). La especificidad y la función de los miRNA están determinados por los nucleótidos 2 a 7 de la región 5' de los miRNA maduros (la llamada región "semilla" del miRNA): dichos nucleótidos deben ser obligatoriamente complementarios al mRNA diana (Lewis et al., 2005). Alrededor de un 60% de los genes que codifican proteínas contienen sitios de unión de miRNAs en sus extremos 3’ UTRs (Friedman RC et al., 2009) y la unión de un solo miRNA no produce efectos significativos sino que hacen falta muchos sitios de unión para activar la respuesta de los miRNA.

1.6.4 MiRNAs y su implicación en la LLC

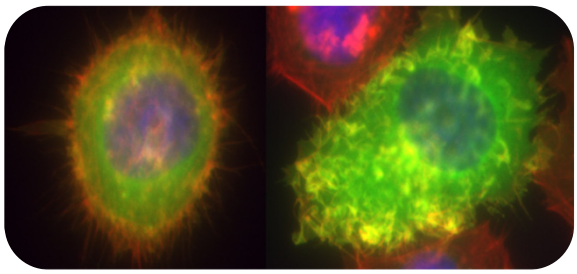
Numerosos estudios de expresión y funcionales han documentado que los miRNAs controlan procesos biológicos críticos, tales como la proliferación y metabolismo celular, apoptosis, hematopoyesis, el desarrollo y la homeostasis de tejidos, además de encontrarse directamente implicados en numerosas patologías, entre ellas la transformación tumoral. Los miRNA pueden funcionar como supresores de tumores o como oncogenes y su desregulación está implicada en la oncogénesis (Esquela-Kerscher et al., 2006).

En la LLC, varios miRNAs han sido encontrados sobre expresados de forma recurrente en comparación con linfocitos B normales (LBN), como el miRNA-155 (Fulci et al., 2007; Marton et al., 2008; Lawrie et al., 2009; Vargova et al., 2011; Zhu et al., 2012; Li et al., 2011), miR-150 (Fulci et al., 2007; Lawrie et al., 2009; Li et al., 2011), miR-101 (Fulci et al., 2007; Zhu et al., 2012; Li et al., 2011), miR-21 (Fulci et al., 2007; Zhu et al., 2012), miR-29a (Zhu et al., 2012; Li et al., 2011), o miR-29c (Lawrie et al., 2009; Li et al., 2011), o infra-expresados, como

miR-181a, miR-181b (Marton et al., 2008; Zhu et al., 2012; Li et al., 2011), y miR-223 (Marton et al., 2008; Lawrie et al., 2009; Li et al., 2011).

Los pacientes con LLC que presentan del(13q) o del(17p) generalmente infra-expresan miR-15a (Smonskey et al., 2012; Rodríguez et al., 2012) o miR-34a (Lawrie et al., 2009; Dijkstra et al., 2009; Zenz et al., 2009; Mraz et al., 2009; Negrini et al., 2014), localizados en la región delecionada respectivamente, en comparación con otros subtipos citogenéticos. Diversos modelos animales han ilustrado el potencial oncogénico de varios miRNAs, incluyendo miR-155, miR-21, miR-29a, o el grupo miR-17~92 (Costinean et al., 2006; Medina et al., 2010; Santanam et al., 2010; Sandhu et al., 2013), y el potencial supresor de tumores de otros como el grupo miR-15a/16-1 (Raveche et al., 2007; Klein et al., 2010). La firma de miRNAs frecuentemente incluye una alta expresión de miR-15a, miR-16, o miR-23b en pacientes que expresan marcadores de peor pronóstico como ZAP-70 (Marton et al., 2008; Mraz et al., 2009; Calin et al., 2005; Papakonstantinou et al., 2013). En pacientes ZAP-70 negativos, miR-29a, miR-29b, miR-29c y miR-223 muestran a menudo niveles más altos de expresión (Marton et al., 2008; Lawrie et al., 2009; Li et al., 2011; Calin et al., 2005; Papakonstantinou et al., 2013).

2. OBJETIVOS



Como parte de esta tesis, se trató de investigar la función de DOCK10, por primera vez, definiendo la especificidad de la proteína completa por las GTPasas Rho "clásicas".

Nuestro grupo identificó a DOCK10 como gen inducible por IL-4 en linfocitos B y en LLC y emprendió su clonaje y estudio de su función, que podría aportar claves en el conocimiento de las actividades biológicas de la IL-4. El conocimiento de su secuencia sugiere que DOCK10 podría funcionar como activador de Rho GTPasas, pero se hace necesario realizar plantear determinados ensayos bioquímicos y generar modelos celulares para definir su especificidad e implicación en fenómenos de remodelado del citoesqueleto de actina y motilidad celular.

Existen distintas hipótesis sobre la patogenia de la LLC pero las causas reales son aún poco conocidas. Mediante la comparación de los perfiles de expresión génica entre LLC y LBN se aportarían nuevos datos útiles para el estudio de los mecanismos moleculares subyacentes a esta patología. Algunos estudios muestran que las LLC sobreexpresan genes de la ruta de la IL-4, sugiriendo que dicha ruta pudiera estar anormalmente activada en la LLC (Klein et al., 2009; Zheng et al., 2002), y que el tratamiento con IL-4 podría ser perjudicial (Lundin et al., 2001). El estudio de la expresión génica inducida por la IL-4 en las LLC podría aportar claves para valorar si la IL-4 juega un papel oncogénico en la LLC. Si se identificasen miRNAs modulados por IL-4, éstos podrían ser a su vez responsables, al menos en parte, de los cambios inducidos por la IL-4. El estudio propuesto contribuiría al esclarecimiento de los mecanismos moleculares de la señalización por la IL-4, lo que podría aprovecharse para buscar alternativas terapéuticas mediante la manipulación de esta vía, con inhibidores específicos o con nuevos abordajes más experimentales como el empleo de miRNA.

Por tanto se plantearon los siguientes objetivos:

1. Investigar la especificidad de DOCK10 para interaccionar con las GTPasas Rho "clásicas", y su papel potencial como GEF activador de estas proteínas.

2. Investigar la función de DOCK10 en la morfología celular, dinámica del citoesqueleto de actina y protrusiones de membrana mediante la generación de un modelo celular basado en su expresión estable e inducible en la línea celular HeLa.

3. Identificar y analizar comparativamente los genes regulados por la IL-4 en leucemias linfocíticas crónicas y linfocitos B normales mediante microarrays de expresión génica, para profundizar en los mecanismos asociados a las funciones de la IL-4 y detectar potenciales alteraciones en la respuesta a la IL-4 asociadas a la leucemia.

4. Analizar comparativamente la respuesta génica a la IL-4 en pacientes con leucemias linfocíticas crónicasagrupados de acuerdo a la expresión del marcador pronóstico ZAP-70, identificar genes candidatos a participar en la función anti-apoptótica de la IL-4,y estudiar la implicación del factor de transcripción NFκB mediante el uso de un inhibidor de NFκB y la realización de microarrays de expresión génica.

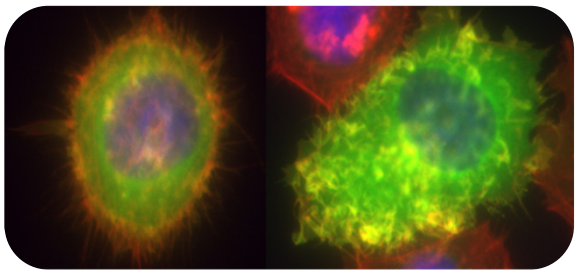
5. Identificar los miRNAs regulados por la IL-4 en leucemias linfocíticas crónicas mediante microarrays de miRNA, para profundizar en los mecanismos asociados a las funciones de la IL-4 y potenciales alteraciones en la leucemia linfocítica crónica.

3. RESULTADOS

3.1 Dock10, a Cdc42 and Rac1 GEF, induces loss of elongation, filopodia, and ruffles in cervical cancer epithelial HeLa cells

3.2 The Gene Expression Response of Chronic Lymphocytic Leukemia Cells to IL-4 Is Specific, Depends on ZAP-70 Status and Is Differentially Affected by an NFκB Inhibitor

3.3 IL-4 Up-Regulates MiR-21 and the MiRNAs Hosted in the CLCN5 Gene in Chronic Lymphocytic Leukemia



3.1

DOCK10, un GEF para Cdc42 y Rac1, induce una pérdida de elongación, filopodios y ondulaciones de membrana en células epiteliales de cáncer de cérvix HeLa

Dock10, a Cdc42 and Rac1 GEF, induces loss of elongation, filopodia, and ruffles in cervical cancer epithelial HeLa cells

Natalia Ruiz-Lafuente, María-José Alcaraz-García, Azahara-María García-Serna, Silvia Sebastián-Ruiz, María-Rosa Moya-Quiles, Ana-María García-Alonso, Antonio Parrado

Biology Open 2015; 4: 627-635

ABSTRACT: Dock10 is one of the three members of the Dock-D family of Dock proteins, a class of guanine nucleotide exchange factors (GEFs) for Rho GTPases. Its homologs Dock9 and Dock11 are Cdc42 GEFs. Dock10 is required for maintenance of rounded morphology and amoeboid-type movement. Full-length isoforms of Dock10 have been recently cloned. Here, we address GTPase specificity and GEF activity of Dock10. In order of decreasing intensity, Dock10 interacted with nucleotide-free Rac1, Cdc42, and Rac3, and more weakly with Rac2, RhoF, and RhoG. Inducible expression of Dock10 in HeLa epithelial cells promoted GEF activity on Cdc42 and Rac1, and a morphologic change in two-dimensional culture consisting in loss of cell elongation, increase of filopodia, and ruffles. Area in contact with the substrate of cells that spread with non-elongated morphology was larger in cells expressing Dock10. Inducible expression of constitutively active mutants of Cdc42 and Rac1 in HeLa cells also induced loss of elongation. However, Cdc42 induced filopodia and contraction, and Rac1 induced membrane ruffles and flattening. When co-expressed with Dock10, Cdc42 potentiated filopodia, and Rac1 potentiated ruffles. These results suggest that Dock10 functions as a dual GEF for Cdc42 and Rac1, affecting cell morphology, spreading and actin cytoskeleton protrusions of adherent HeLa cells.

URL: http://bio.biologists.org/content/4/5/627.long

3.2

La expresión génica en respuesta a la IL-4 en la Leucemia Linfocítica Crónica es específica, depende del status de ZAP-70 y se ve afectada diferencialmente por un inhibidor de NFκB

The Gene Expression Response of Chronic Lymphocytic Leukemia Cells to IL-4 Is Specific, Depends on ZAP-70 Status and Is Differentially Affected by an NFκB Inhibitor

Natalia Ruiz-Lafuente, María-José Alcaraz-García, Silvia Sebastián-Ruiz, Joaquín Gómez-Espuch, Consuelo Funes, José-María Moraleda, María-Carmen García-Garay, Natividad Montes-Barqueros, Alfredo Minguela, María-Rocío Álvarez-López, Antonio Parrado

PLoS ONE 2014; 9: e109533

ABSTRACT: Interleukin 4 (IL-4), an essential mediator of B cell development, plays a role in survival of chronic lymphocytic leukemia (CLL) cells. To obtain new insights into the function of the IL-4 pathway in CLL, we analyzed the gene expression response to IL-4 in CLL and in normal B cells (NBC) by oligonucleotide microarrays, resulting in the identification of 232 non-redundant entities in CLL and 146 in NBC (95 common, 283 altogether), of which 189 were well-defined genes in CLL and 123 in NBC (83 common, 229 altogether) ($p<0.05$, 2-fold cut-off). To the best of our knowledge, most of them were novel IL-4 targets for CLL (98%), B cells of any source (83%), or any cell type (70%). Responses were significantly higher for 54 and 11 genes in CLL and NBC compared to each other, respectively. In CLL, ZAP-70 status had an impact on IL-4 response, since different sets of IL-4 targets correlated positively or negatively with baseline expression of ZAP-70. In addition, the NFκB inhibitor 6-Amino-4-(4-phenoxyphenethylamino)quinazoline, which reversed the anti-apoptotic effect of IL-4, preferentially blocked the response of genes positively correlated with ZAP-70 (e.g. CCR2, SUSD2), but enhanced the response of genes negatively correlated with ZAP-70 (e.g. AUH, BCL6, LY75, NFIL3). Dissection of the gene expression response to IL-4 in CLL and NBC contributes to the understanding of the anti-apoptotic response. Initial evidence of a connection between ZAP-70 and NFκB supports further exploration of targeting NFκB in the context of the assessment of inhibition of the IL-4 pathway as a therapeutic strategy in CLL, especially in patients expressing bad prognostic markers.

URL: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0109533

3.3

La IL-4 regula positivamente miR-21 y los miRNAs alojados en el gen CLCN5 en la Leucemia Linfocítica Crónica

IL-4 Up-Regulates MiR-21 and the MiRNAs Hosted in the CLCN5 Gene in Chronic Lymphocytic Leukemia

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ABSTRACT: Interleukin 4 (IL-4) induces B-cell differentiation and survival of chronic lymphocytic leukemia (CLL) cells. MicroRNAs (miRNAs) regulate mRNA and protein expression, and several miRNAs, deregulated in CLL, might play roles as oncogenes or tumor suppressors. We have studied the miRNA profile of CLL, and its response to IL-4, by oligonucleotide microarrays, resulting in the detection of a set of 129 mature miRNAs consistently expressed in CLL, which included 41 differentially expressed compared to normal B cells (NBC), and 6 significantly underexpressed in ZAP-70 positive patients. IL-4 stimulation brought about up-regulation of the 5p and 3p mature variants of the miR-21 gene, which maps immediately downstream to the VMP1 gene, and of the mature forms generated from the miR-362 (3p and 5p), miR-500a (3p), miR-502 (3p), and miR-532 (3p and 5p) genes, which map within the third intron of the CLCN5 gene. Both genes are in turn regulated by IL-4, suggesting that these miRNAs were regulated by IL-4 as passengers from their carrier genes. Their levels of up-regulation by IL-4 significantly correlated with cytoprotection. MiR-21 has been reported to be leukemogenic, associated to bad prognosis in CLL, and the miRNA more frequently overexpressed in human cancer. Up-regulation by IL-4 of miR-21 and the miRNAs hosted in the CLCN5 locus may contribute to evasion of apoptosis of CLL cells. These findings indicate that the IL-4 pathway and the miRNAs induced by IL-4 are promising targets for the development of novel therapies in CLL.

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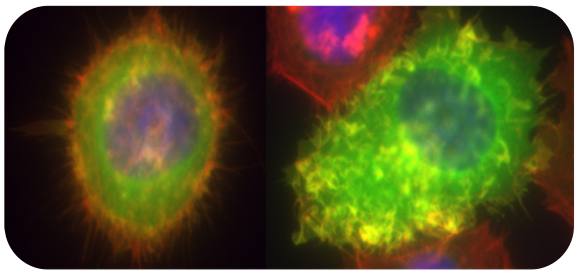
La aportación de la doctoranda Dª **Natalia Ruiz Lafuente** en los artículos anteriormente citados se explican a continuación:

En la publicación: “Dock10, a CDC42 and Rac1 GEF, induces loss of elongation, filopodia, and ruffles in cervical cancer epithelial HeLa cells”, siendo la responsable de la generación de clones estables con expresión inducible, realización de los ensayos de interacción y activación, análisis por western blot y cultivo celular para la toma de imágenes por microscopía y contribuyendo de forma significativa a la discusión de los resultados obtenidos y a la redacción de dicho artículo.

En la publicación: “The gene expression response of chronic lymphocytic leukemia cells to IL-4 is specific, depends on ZAP-70 status and is differentially affected by an NFκB inhibitor” siendo la responsable de la toma de muestras, aislamiento de poblaciones celulares y su cultivo, aislamiento de ARN y realización de análisis por microarrays de expresión génica, validación por PCR cuantitativa, análisis estadístico de los datos obtenidos y contribuyendo de forma significativa a la discusión de los resultados obtenidos y a la redacción de dicho artículo.

En la publicación: “IL-4 up-regulates MiR-21 and theMiRNAs hosted in the CLCN5 gene in chronic lymphocytic leukemia” siendo la responsable de la toma de muestras, aislamiento de poblaciones celulares y su cultivo, aislamiento de ARN y realización de análisis por microarrays de miRNA, validación por PCR cuantitativa y análisis estadístico de los datos obtenidos y contribuyendo de forma significativa a la discusión de los resultados obtenidos y a la redacción de dicho artículo

4. DISCUSIÓN GENERAL



La dinámica del citoesqueleto de actina es regulada a través de la señalización por pequeñas GTPasas de la familia Rho. En un escrutinio por silenciamiento génico de GEFs inductores de cambios morfológicos en células de melanoma, se identificó que la proteína DOCK10 funcionaba como GEF sobre Cdc42 (Gadea et al., 2008). Sin embargo, definir la especificidad de DOCK10 por las GTPasas se ha visto dificultado por la carencia de vectores de expresión de la proteína DOCK10 completa. Nuestro grupo ha clonado dos isoformas completas de DOCK10 con primer exón alternativo, que hemos denominado DOCK10.1 y DOCK10.2 (Alcaraz-García et al., 2011). Dichos clonajes hacen posible determinar la especificidad de DOCK10 por las GTPasas Rho. En la Publicación 3.1, se presentan evidencias de que ambas isoformas interaccionan *in vitro* con las GTPasas Cdc42 y Rac1 en condiciones libres de carga de nucleótido, y que estas interacciones conducen a un incremento en los niveles de activación de Cdc42 y Rac1 en células epiteliales HeLa, derivadas de un carcinoma de cérvix. Por lo tanto, DOCK10, que comparte menor homología de secuencia con DOCK9 y DOCK11, los otros dos miembros de la subfamilia Zizimin o DOCK-D de las proteínas DOCK, y además es más extensa en unos 100 aminoácidos, también difiere en su especificidad por las GTPasas, puesto que ambas están definidas como específicas para Cdc42 (Meller et al., 2002; Nishikimi et al., 2005; Lin et al., 2006), y nuestros ensayos de activación *in vitro* también apoyan las conclusiones de los estudios referenciados, puesto que muestran cómo DOCK9 y DOCK11 interaccionan principalmente con Cdc42 en condiciones libres de carga de nucleótido. DOCK10 no es el único caso entre las proteínas DOCK que tienen doble especificidad por proteínas Rac y Cdc42, pues también la tienen DOCK6 (Miyamoto et al., 2007) y DOCK7 (Watabe-Uchida et al., 2006; Zhou et al., 2013), mientras que el resto de proteínas DOCK presentan especificidad por una de las dos GTPasas: de DOCK1 hasta DOCK5 por proteínas Rac (Kiyokawa et al., 1998; Nishihara et al., 2002; Kulkarni et al., 2011; Namekata et al., 2004; Hiramoto et al., 2006; Vives et al., 2011) y DOCK8, como DOCK9 y DOCK11, por Cdc42 (Harada et al., 2012).

En la Publicación 3.1, se presentan los resultados del estudio de la función de la isoforma DOCK10.1 por medio de inducir su sobre-expresión en células HeLa cultivadas en medio líquido, las cuales proliferan adheridas a la superficie plana, bien sea plástico de cultivo de tejidos, bien soporte de vidrio cubierto de colágeno o poli-lisina. Las células se transfectaron de manera estable con un vector de expresión inducible en ausencia de doxiciclina (dox) que además introduce una etiqueta HA en el extremo N-terminal de DOCK10.1, y se seleccionaron clones con expresión inducible de esta proteína HA-DOCK10.1. Nuestros hallazgos principales son que HA-DOCK10.1 induce un cambio morfológico desde una forma elongada y poligonal, propia de las células HeLa, hasta una forma más redondeada, no

poligonal, aumentando moderadamente su área de contacto con el substrato, y en las células se incrementa de manera evidente la presencia de filopodios y ondulaciones de membrana dorsales y en el borde celular. La pérdida de elongación es concordante con los resultados previos publicados por Gadea et al. (2008), que mostraron que el silenciamiento génico de DOCK10 mediante miRNA induce el efecto opuesto, es decir, un cambio de una forma redondeada hacia elongada, en cultivo tridimensional semisólido en una matriz de colágeno (Gadea et al., 2008). En nuestro estudio, otros clones seleccionados de HeLa con expresión inducible de mutantes constitutivamente activos de Cdc42 y Rac1 etiquetadas con la proteína fluorescente EGFP (EGFP-Cdc42L61 y EGFP-Rac1L61), también pierden la forma elongada poligonal. Además, la pérdida de elongación se potenciamediante la co-expresión de HA-DOCK10.1 y EGFP-Cdc42 o EGFP-Rac1, especialmente el primero. Estas observaciones concuerdan con otras realizadas en fibroblastos con deleción de Cdc42, que presentan forma de huso (Czuchra et al., 2005), y con deleción de Rac1, que presentan forma elongada y son defectivos en formación de lamellipodia y ondulaciones de membrana (Vidali et al., 2006). En conclusión, DOCK10.1 podría inducir pérdida de elongación celular a través de Cdc42 y/o de Rac1.

El cambio morfológico inducido por HA-DOCK10.1 no implica un cambio global en el área celular en contacto con la superficie, aunque las células no elongadas producidas en ausencia de dox presentaron una expansión mayor que las células no elongadas en presencia de dox, sugiriendo que la expresión de HA-DOCK10.1 inducía la expansión del área de contacto. La literatura indica que Cdc42 y Rac1 pueden jugar un papel en la extensión celular (Price et al., 1998; Wells et al., 2004; Czuchra et al., 2005). Nuestros resultados muestran que EGFP-Cdc42L61, constitutivamente activo, no induce un cambio global significativo en la extensión celular, yla doble expresión de HA-DOCK10.1 y EGFP-Cdc42L61, tampoco. Sin embargo, EGFP-Rac1L61, constitutivamente activo, potencia la expansión de la superficie celular, y esto también ocurrióen el caso de la doble expresión de HA-DOCK10.1 y EGFP-Rac1L61. Constatamos que los clones pueden tener expresión residual en presencia de dox, que podría generar diferencias basales. Así, de todos nuestros clones, las células con doble expresión de HA-DOCK10.1 y EGFP-Rac1L61 son las que presentan una mayor extensión en presencia de dox, posiblemente debido a la expresión residual de EGFP-Rac1L61 y HA-DOCK10.1. En conjunto, nuestos resultados sugieren que la mayor capacidad de expansión de las células no elongadas inducida por DOCK10.1 podría ser mediada a través de Rac1.

HA-DOCK10.1 expresado en las células HeLa potenció la actividad de filopodios y ondulaciones de membrana, que son protrusiones celulares inducidas por la activación de Cdc42 y Rac1, respectivamente (Wennerberg y Der, 2004; Aspenström et al., 2004; Chhabra y

Higgs, 2007; Heasman y Ridley, 2008). En la Publicación 3.1 se ha verificado que EGFP-Cdc42L61 inducía filopodios, y EGFP-Rac1L61, ondulaciones de membrana. En los clones con expresión doble, los mutantes constitutivamente activos EGFP-Cdc42L61 y EGFP-Rac1L61 ejercen un papel dominante, puesto que el doble expresor de HA-DOCK10.1 y EGFP-Cdc42L61mostró exacerbación de los filopodios y reducción de las ondulaciones de membrana respecto de HA-DOCK10.1 sólo, y el doble expresor de HA-DOCK10.1 y EGFP-Rac1L61, exacerbación de las ondulaciones de membrana y reducción de filopodios respecto de HA-DOCK10.1 sólo.

En resumen, usando un sistema de expresión génica inducible en una línea tumoral adherente, presentamos aquí el primer modelo celular basado en la sobre-expresión para estudiar la función de DOCK10. Nuestros datos avalan el papel de DOCK10 en la pérdida de elongación celular y activación de Cdc42 que se habían sugerido en estudios anteriores, y apoya nuevas funciones en la activación de Rac1, inducción de filopodios y ondulaciones de membrana. Nuestras líneas transfectantes estables para la expresión de HA-DOCK10.1 podrían ser útiles para investigar la señalización celular de este regulador de las pequeñas GTPasas Rho y su función en diferentes condiciones del micro medio ambiente biológico.

DOCK10 es un elemento de un cuadro más amplio que es el programa de regulación génica inducido por la IL-4, una citoquina que juega un papel central en el desarrollo y diferenciación de los linfocitos T y B (Okada et al., 2003). En estudios previos, nuestro grupo identificó DOCK10 como un gen cuya expresión se inducía en LBN y malignos de LLC en respuesta a IL-4 *in vitro* (Yelo et al., 2008; Alcaraz-García et al., 2011). Sin embargo, el programa completo de regulación génica por la IL-4 en LBN y LLC no había sido estudiado de modo exhaustivo hasta ahora, a pesar de que la IL-4 ha sido reconocida como un factor de supervivencia clave en la LLC durante mucho tiempo (Dancescu et al., 1992). Proporcionada por los linfocitos T en sus nichos microambientales *in vivo* (los pseudofolículos de los ganglios linfáticos), la IL-4 podría sostener la proliferación celular, la leucemogénesis y su progresión, como otros autores han sugerido en linfoma folicular (Pangault et al., 2010). En la Publicación 3.2, se identificaron 229 genes bien definidos como regulados por la IL-4 en LLC y LBN en conjunto, la mayor parte de los cuales son nuevas dianas de la IL-4 en LLC, células B de cualquier procedencia, linfocitos u otros tipos celulares en los que se haya estudiado la respuesta génica a la IL-4 (Schroder et al., 2002; Chen et al., 2003; Lee et al., 2004; Lund et al., 2005; Lu et al., 2005; Chaitidis et al., 2005; Zhang et al., 2008). Los genes regulados por la IL-4 previamente conocidos proporcionan una validación a nuestro estudio. Una validación adicional fue obtenida para un grupo amplio de nuevos genes identificados mediante PCR cuantitativa en tiempo real. La introducción de dos muestras de referencia (a tiempo 0, “Pre”,

y el cultivo sin IL-4, "Ctrl") en el diseño experimental contribuyó a definir de manera precisa los genes regulados por la IL-4 mediante exclusión de aquellos modulados por el cultivo celular. La similitud en los resultados del análisis de ANOVA usando las tres condiciones Pre, Ctrl e IL-4 y del análisis de la t de Student comparando Ctrl e IL-4 mostró que la comparación IL-4 vs Ctrl, además de necesaria, era suficiente para la identificación de la mayoría de los genes regulados por la IL-4.

El número de genes regulados por la IL-4 en LLC fue mayor que en LBN. Este resultado puede tener dos explicaciones, no excluyentes: primera, el mayor tamaño de la muestra de LLC (n=23 vs n=13); y segunda, una respuesta génica más intensa de las LLC a la IL-4. Una parte sustancial de la respuesta génica fue común a las LLC y los LBN, cualitativa y cuantitativamente. Sin embargo, grupos de 54 y 11 genes con respuestas diferentes (la mayor parte específicos) se encontraron en LLC y LBN, respectivamente. Estudios previos han mostrado una expresión aumentada del receptor para la IL-4 en LLC, a nivel de proteína (Douglas et al. 1997) y a nivel de mRNA mediante microarrays (Zheng et al., 2002), aunque en otro estudio no se encontraron diferencias (Kaminski et al., 1998). En nuestro estudio, como en el último, no encontramos diferencias significativas entre los niveles basales de mRNA para IL4R entre LLC y LBN. Los dos primeros estudios fueron realizados con pocas muestras y/o usaron células B de diferente origen (amígdalas). En nuestro estudio, la expresión de IL4R se indujo por IL-4 con similar intensidad en LLC y LBN, sugiriendo que la diferente respuesta génica global a la IL-4 en LLC y NBC es independiente de los niveles del receptor de la IL-4, y, por tanto, surge aguas debajo del mismo.

Encontrar correlaciones entre los genes regulados y marcadores pronósticos utilizados en la práctica clínica podría ayudar a revelar nuevas rutas alteradas y nuevas dianas terapéuticas alternativas a las actuales. En la Publicación 3.2 se observó la existencia de dos grupos de genes regulados por la IL-4 cuyos cambios se correlacionaron positiva y negativamente, respectivamente, con los niveles basales del marcador pronóstico ZAP-70. Es decir, un grupo de genes se induce con mayor intensidad en pacientes ZAP-70+ y otro grupo se induce con mayor intensidad en pacientes ZAP-70–. El grupo de genes correlacionado positivamente con ZAP-70 es mayor. ZAP-70 no había sido relacionado con la ruta de la IL-4 anteriormente, pero sí se habían comunicado varias interacciones con componentes de esta ruta (Figura 6 de la publicación 3.2). Los genes regulados diferencialmente juegan papeles esenciales en rutas de desarrollo y supervivencia, tales como la ruta de WNT y la adhesión celular en el grupo ZAP-70+ (CDH1, WNT5B, WNT11). Se había publicado que la expresión de CDH1 estaba reprimida epigenéticamente en la LLC (Moskalev et al., 2012), y nuestros datos sugieren que la IL-4 libera esta represión, especialmente en pacientes ZAP-70+. Estos datos

podrían estar relacionados con un estudio que muestra que las LLC ZAP-70+ tienen mayor capacidad adhesiva a las células estromales en respuesta a CD40L+IL-4 (Lafarge et al., 2014). Estudios previos habían detectado niveles aumentados de las proteínas anti-apoptóticas BCL2, usando IL-4 (Dancescu et al., 1992), o MCL1, BCL2L1, BCL2A1, y XIAP, usando el sistema CD40L/IL-4 (Willimott et al., 2007; Cosimo et al., 2013), sin correlación a nivel de mRNA, y en nuestro estudio usando sólo IL-4 no se detectaron cambios significativos en la expresión de sus tránscritos. No obstante, nuestro estudio proporciona varios genes candidatos para el mecanismo anti-apoptótico de la IL-4 en LLC, para la mayor sensibilidad de las LLC en cultivo celular comparado con los LBN, y para el mayor efecto protector de la IL-4 en LLC comparado con LBN. Algunos de los genes diana de la IL-4, y algunos de los genes que no siendo diana se encontraron alterados por el cultivo pero estabilizados por la IL-4, habían sido previamente relacionados con la apoptosis, y sus respuestas fueron frecuentemente más potentes en LLC (p. ej. CASP3, GFI1, ICAM1, LNPEP, NCF2, NFKBIZ, RPS6KA2, GADD45B). Además, varias dianas de la IL-4, algunas de la cuales no habían sido previamente relacionadas con la IL-4, se correlacionaron con el grado de citoprotección. Los más aumentados de esta lista, HOMER2 y BCL6, también tuvieron respuestas más aumentadas en LLC. HOMER2 pertenece a una familia de proteínas de andamiaje que impiden la apoptosis neuronal a través de PI3K y el receptor de glutamato (Rong et al., 2003), y regulan la activación de las células T mediante unión a NFAT (Huang et al., 2008), pero su papel en células B no ha sido explorado. BCL6 es un factor represor de la transcripción asociado a un peor pronóstico en LLC (Jantus Lewintre et al., 2009). La identificación de estos genes supone un punto de partida para futuros estudios cuyo objetivo sería definir con precisión el mecanismo de supervivencia desencadenado por la IL-4. En contraste con el estudio de Coscia et al. (2011) que indica la menor sensibilidad a apoptosis espontánea de la LLC que expresan marcadores de mal pronóstico, en la Publicación 3.2 no se observan diferencias significativas en pacientes ZAP-70+ y ZAP-70−, a pesar de la regulación diferencial de varios genes diana de la IL-4 relacionados con función apoptótica (SOCS1, NFKBIZ, LNPEP, RPS6KA2). No obstante, sí se observa una citoprotección diferencial después de tratamiento con IL-4 y un inhibidor de NFκB (ver más adelante).

NFκB y ZAP-70 son efectores de la vía de señalización del receptor de células B (RCB). La expresión de NFκBse induce como consecuencia de la activación del RCB, y se asocia a supervivencia celular y expresión de ZAP-70 en LLC (López-Guerra et al., 2009). ZAP-70 potencia la respuesta producida por la activación del RCB en LLC (Chen et al., 2008; Calpe et al., 2011). Los inhibidores de NFκB inducen apoptosis en LLC, y los pacientes ZAP-70+ podrían tener una actividad de NFκB y una sensibilidad a la apoptosis aumentadas (Pickering et al., 2007; Hewamana et al., 2008). En la Publicación 3.2 se muestra que un inhibidor de NFκB

contrarrestó el efecto anti-apoptótico de la IL-4, especialmente en pacientes ZAP-70+, y que la respuesta génica de la mayor parte de los genes diana de la IL-4 correlacionados positivamente con los niveles basales de ZAP-70 depende de NFκB. Por el contrario, el inhibidor de NFκB potenció la respuesta del grupo de genes que se correlacionaba negativamente con los niveles basales de ZAP-70, sugiriendo un nuevo mecanismo mediante el cual ZAP-70 y NFκB actuarían juntos disminuyendo la respuesta de este grupo de genes a la IL-4. Ejemplos de genes de ambos grupos fueron confirmados por PCR cuantitativa, tales como CCR2, que es el receptor de la quimiocina CCL2, implicada en la supervivencia celular en LLC (Burguess et al., 2012), NFIL3, un factor de transcripción pro-surpervivencia en células B (Ikushima et al., 1997), y BCL6. La puesta en evidencia de este mecanismo ha sido posible mediante la realización de estudios a nivel transcriptómico. La búsqueda de una explicación molecular, incluido el papel jugado por ZAP-70, será el objetivo de estudios futuros. El inhibidor de la quinasa JAK3 PF-956980 (Steele et al., 2010) bloquea la activación de STAT6 y podría abolir la respuesta génica a la IL-4. Como el inhibidor de NFκB, PF-956980 contrarresta la citoprotección por IL-4 en la LLC *in vitro*, y ofrece un potencial terapéutico que está siendo investigado en la actualidad. Inhibir la ruta de la IL-4 podría ser una estrategia terapéutica válida en la LLC, y la vía podría bloquearse a distintos niveles. Puesto que la inhibición de NFκB contrarresta la citoprotección de la IL-4, y se asocia a una respuesta atenuada de un grupo de genes diana de la IL-4, la inhibición de NFκB debería ser explorada con mayor profundidad especialmente en pacientes que expresan marcadores de mal pronóstico.

Los miRNAs son un grupo especial de genes de RNA, que regulan la expresión de genes convencionales mediante apareamiento completo o parcial con sitios complementarios en los mRNAs dianas, normalmente en sus regiones 3' no codificantes. Como consecuencia, la expresión génica se regula negativamente a través de degradación del mRNA o, más usualmente, de represión traduccional. Un sólo miRNA podría reprimir la expresión de hasta varios cientos de genes (Di Leva et al., 2014). En la Publicación 3.3, se estudió mediante microarrays la regulación por IL-4 de los perfiles de expresión de 955 miRNAs maduros en LLC. En primer lugar, se identificaron 129 miRNAs expresados significativamente en LLC. Los expresados con niveles más altos coincidieron con los de estudios previos, y fueron similares en LLC y LBN, aunque se pusieron en evidencia algunas diferencias entre ambos, por ejemplo las ya conocidas sobre-expresiones en LLC de miR-150-5p, miR-29a-3p, miR-155-5p, o miR-101-3p, e infra-expresiones de miR-181a-5p, o miR-181b-5p (Fulci et al., 2007; Marton et al., 2008; Lawrie et al, 2009; Vargova et al., 2011; Zhu et al., 2012; Li et al., 2011), y otras diferencias no establecidas de manera firme aún, tales como las sobre-expresiones de miR-451a, miR-28-5p,

miR-144-5p, miR-486-5p, o miR-486-3p, y las infra-expresiones de miR-126-3p, miR-365a-3p, miR-199a-3p, o miR-582-5p.

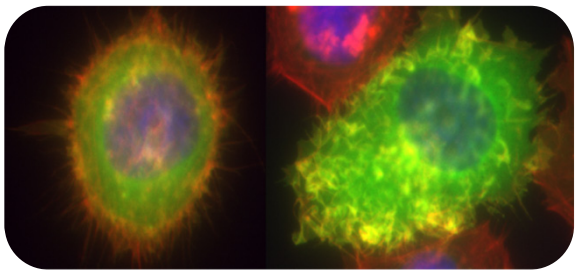
El análisis no supervisado de agrupamiento jerárquico agrupó las muestras Pre (a tiempo 0), y las muestras Ctrl e IL-4 mezcladas entre ellas, ilustrando que el cultivo celular, por sí mismo, induce cambios, como habíamos observado previamente al analizar la expresión génica convencional. Las muestras Ctrl e IL-4 se agruparon moderadamente de acuerdo al status de ZAP-70, indicando que este marcador tiene un impacto significativo sobre la expresión de los miRNAs en LLC. Concretamente, se encontraron infra-expresados en pacientes ZAP-70+, los siguientes: miR-29c, en sus variantes 3p y 5p, observación recurrente en los anteriores estudios; miR-146b-5p, como en el estudio de Negrini et al. (2014), y miR-210-3p. Estos resultados apoyan su uso como potenciales biomarcadores pronósticos.

Los análisis de correlación entre los niveles basales de los miRNAs y la apoptosis espontánea identificaron un número relativamente alto de miRNAs candidatos para desarrollar un papel anti- o pro-apoptótico. De esta lista, la mayoría requerirían validación funcional, utilizando una estrategia similar a la utilizada por Bomben et al. (2012) para demostrar el efecto citoprotectivo de miR-17-5p. Puesto que los miRNAs controlan la expresión génica, es posible que los miRNAs regulados por la IL-4 contribuyan a los cambios en la expresión génica y a la función anti-apoptótica. En la Publicación 3.3, se encontró un aumento de la expresión de miR-21 (5p y 3p), miR-362 (3p y 5p), miR-500a-3p, miR-502-3p, y miR-532 (3p y 5p) en LLC. Todos estos miRNAs incrementaron su expresión también en al menos una de las tres muestras de LBN, indicando que su regulation por IL-4 no es exclusiva de las LLC.

MiR-21 fue previamente encontrado sobre-expresado en tejido pulmonar alérgico en modelos murinos de asma, tales como transgénicos para IL-4 e IL-13, ratones tratados con alérgeno, IL-4 o IL-13 (Lu et al., 2009), y se inducía por IL-4 en células B (Thapa et al., 2012). MiR-21 se produce a partir de dos tipos de tránscritos primarios de miR-21, un pri-miR-21 que se transcribe a partir de un promotor localizado en los últimos intrones del gen VMP1, y un tránscrito VMP1-miR-21 que se inicia a partir del promotor de VMP1, y en ambos casos se saltan las señales de poliadenilación de VMP1 (Ribas et al., 2012). Puesto que la expresión de VMP1 se induce por IL-4, según la Publicación 3.2, se puede hipotetizar que la regulación positiva de miR-21 por IL-4 es muy probablemente producida a través de la transcripción de VMP1-miR-21. La regulación positiva de los miRNAs contenidos en el tercer exón del gen CLCN5 sugiere que dichos miRNAs aumentaron como pasajeros del tránscrito de CLCN5, otro de los genes diana de la IL-4 previamente definidos en la Publicación 3.2.

MiR-21 es el miRNA más recurrentemente encontrado sobre-expresado en cáncer en humanos (Pan et al., 2010), y su papel oncogénico ha sido demostrado (Medina et al., 2010). Se ha encontrado sobre-expresado en LLC (Fulci et al., 2007; Zhu et al., 2012), asociado con hipometilación de su promotor (Baer et al, 2012). La expresión alta de miR-21 se ha asociado con mal pronóstico y resistencia a quimioterapia en LLC (Rossi et al., 2010; Ferracin et al., 2010) y en cáncer en general (Wang W et al., 2014). A nivel celular, se ha propuesto un rol anti-apoptótico para miR-21, a través de la represión de diversas dianas pro-apoptóticas, tales como PDCD4 y PTEN, resultando en la activación de vías anti-apoptóticas tales como las de Ras y NFκB (Buscaglia y Li, 2011). Los datos que aquí se muestran indican que miR-21-5p y algunos miRNA portados por CLCN5 se correlacionaron significativamente con citoprotección por IL-4 en LLC, apoyando que estos miRNAs sean anti-apoptóticos. Esta hipótesis tendría que ser más directamente probada en estudios futuros. Algunos de los miRNAs alojados en el gen CLCN5 también han sido relacionados con cáncer: por ejemplo, la expression alta de miR-362 se asocia a mal pronóstico y resistencia a apoptosis en cáncer colorrectal y gástrico (Christensen et al., 2013; Xia et al., 2014), la de miR-500a a carcinoma hepatocelular (Yamamoto et al., 2009), y la de miR-502 en cáncer de mama y colon (Song et al., 2009; Zhai et al., 2013). En resumen, la Publicación 3.3 ha permitido identificar no sólo cuales son los miRNAs regulados por la IL-4 sino también los probables mecanismos responsables, contribuyendo a la comprensión de la respuesta anti-apoptótica a la IL-4, que a su vez podría tener importancia en la evasión de la apoptosis en la LLC, la resistencia a quimioterapia, y en definitiva, la leucemogénesis. Estos datos indican que la vía de la IL-4 y los genes y miRNAs inducidos por esta citoquina son dianas prometedoras para el desarrollo de nuevas terapias en la LLC.

5. CONCLUSIONES



1. DOCK10.1 y DOCK10.2 interaccionan *in vitro* de forma específica con las GTPasas Cdc42 y Rac1 en condición libre de carga de nucleótido. DOCK10.1 incrementa los niveles de activación de ambas GTPasasen células epiteliales HeLa.

2. DOCK10.1 induce cambios morfológicos en células HeLa desde una forma elongada y poligonal, propia de dicha línea celular, hasta una forma más redondeada, no poligonal, con incremento de filopodios y ondulaciones de membrana dorsales y en el borde celular. Dichos cambios son coherentes con la activación simultánea de Cdc42 y Rac1, pues evocan aquellos inducidos por las formas constitutivamente activas Cdc42L61 y Rac1L61, es decir, inducción de filopodios y ondulaciones de membrana, respectivamente, y pérdida de la forma elongada en ambos casos, cambios que además se potencian mediante co-expresión de estas formas con DOCK10.1. Los datos sugieren que el mecanismo a través del cual DOCK10.1 induce estos cambios morfológicos es la activación de dichas GTPasas, enunciada en la primera conclusión.

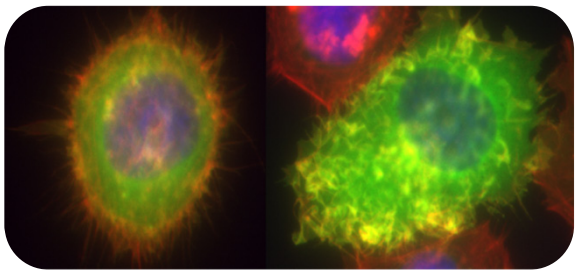
3. La IL-4 regula la expresión de al menos 229 genes en linfocitos B, 189 en leucemias linfocíticas crónicas y 123 en linfocitos B normales, siendo 89 comunes. La mayoría de estos genes son inducidos, y en valores absolutos los cambios son más significativos que los de los genes reprimidos. Esta regulación no era conocida previamente para la mayor parte de los mismos. En general los cambios son más intensos en las leucemias linfocíticas crónicas, sugiriendo que esta ruta es potencialmente muy activa en dichas células, aunque no parece deberse a alteración de los niveles del receptor de la IL-4. Así, hay grupos de 54 genes y 11 genes regulados con mayor intensidad en leucemias linfocíticas crónicas y en linfocitos B normales, respectivamente, con enriquecimiento en funciones tales como la ruta de señalización de WNT y adhesión celular, en las primeras, y la ruta de CREB y la angiogénesis, en los segundos.

4. Existen genes regulados diferencialmente por la IL-4 en pacientes ZAP-70+ y ZAP-70−, con enriquecimiento en la ruta de señalización de WNT y adhesión celular, en los primeros, y en estrés oxidativo y angiogénesis, en los segundos. También hay genes que se correlacionan con la protección anti-apoptótica de la IL-4, tales como HOMER2 y BCL6, que podrían ser candidatos para dicho mecanismo. La respuesta diferencial a la IL-4 en pacientes ZAP-70+ y ZAP-70− depende, al menos en parte, de la función del factor de transcripción NFκB, como sugiere el uso de un inhibidor deNFκB, que atenúa

la respuesta de genes preferentemente inducidos por la IL-4 en pacientes ZAP-70+ y en cambio potencia aún más la de genes preferentemente inducidos en pacientes ZAP-70–. Además, la conexión entre NFκB y ZAP-70 también juega un papel en apoptosis, pues el inhibidor de NFκB contrarresta el efecto anti-apoptótico de la IL-4 más significativamente en pacientes ZAP-70+.

5. Existen 8 miRNAs maduros, miR-21 (5p y 3p), miR-362 (3p y 5p), miR-500a-3p, miR-502-3p, y miR-532 (3p y 5p), que se inducen por la IL-4 en leucemias linfocíticas crónicas, probablemente por un mecanismo “pasivo”, siendo miR-21 intragénico a VMP1, el resto de miRNAs intragénicos a CLCN5, y ambos genes inducidos por la IL-4, de modo que los miRNAs se inducirían incrustados dentro de los tránscritos primarios de ambos. Puesto que la IL-4 juega un papel crucial en la evasión de la apoptosis y la resistencia a quimioterapia, la identificación de los genes y miRNAs regulados por la IL-4 permite profundizar en sus mecanismos, y a su vez podrían representar dianas para el desarrollo de nuevas terapias en la leucemia linfocítica crónica.

6. RESUMEN



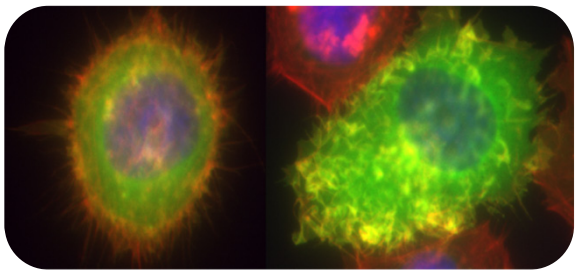
En estudios previos, nuestro grupo identificó a DOCK10 como un gen cuya expresión se induce por IL-4 en linfocitos B normales (LBN) y en leucemias linfocíticas crónicas (LLC). Dada su pertenencia a la familia de proteínas DOCK, se sugiere un posible papel como regulador de las GTPasas Rho. En la publicación 3.1 tratamos de responder algunas preguntas esenciales sobre la función de DOCK10, tales como: (1) su especificidad para interaccionar con las GTPasas Rho "clásicas", que abordamos mediante la realización de ensayos "pulldown" tras transfección transitoria en la línea celular 293T; (2) su papel potencial como GEF activador de estas proteínas, que también abordamos mediante ensayos "pulldown" pero realizados en este caso en un sistema de clones con expresión estable e inducible generado en la línea celular HeLa; y (3) su papel en la morfología celular, dinámica del citoesqueleto de actina y protrusiones de membrana mediante microscopía de fluorescencia en este mismo sistema. Pudimos demostrar que las isoformas de primer exon mutuamente excluyente DOCK10.1 y DOCK10.2 interaccionan in vitro de forma específica con las GTPasas Cdc42 y Rac1 en condición libre de carga de nucleótido y que DOCK10.1 incrementa los niveles de activación de ambas GTPasas. DOCK10.1 induce la transición desde una forma elongada y poligonal, hasta una forma más redondeada, no poligonal, con incremento de filopodios y ondulaciones de membrana, cambios compatibles con la activación de dichas GTPasas.

La patogenia de la LLC es poco conocida, y la IL-4, como factor de crecimiento de los linfocitos B, podría jugar un papel. La IL-4 ejerce un papel anti-apoptótico en la LLC, y algunos estudios sugieren la hipótesis de que podría jugar un papel oncogénico. Puesto que una parte importante de la acción de la IL-4 es muy probablemente ejercida a través de cambios inducidos en la expresión génica, y ésta no ha sido suficientemente estudiada en LBN y LLC, planteamos un análisis mediante microarrays en ambos tipos celulares (publicación 3.2). La IL-4 regula la expresión de al menos 229 genes, 189 en LLC y 123 en LBN, siendo 89 comunes, la mayoría inducidos, y con mayor diferencia que los genes reprimidos, y también mayor intensidad en LLC, sugiriendo que esta ruta es muy activa en esta patología. La mayoría de estos genes no eran dianas conocidas de la IL-4. Además la respuesta génica a la IL-4 es diferente en función de la expresión del factor pronóstico ZAP-70, a través de un mecanismo que implica al factor de transcripción NFκB. También identificamos genes que se correlacionan con la protección anti-apoptótica, tales como HOMER2 y BCL6.

En la publicación 3.3 estudiamos mediante microarrays la respuesta a la IL-4 de un tipo de genes no convencionales denominados miRNAs, importantes en la regulación de la expresión proteica. Identificamos 8 miRNAs maduros, miR-21 (5p y 3p), miR-362 (3p y 5p), miR-500a-3p, miR-502-3p, y miR-532 (3p y 5p), que se inducen por la IL-4 en LLC, y esto ocurre probablemente por un mecanismo "pasivo", siendo miR-21 intragénico a VMP1, el resto de

miRNAs intragénicos a CLCN5, y ambos genes inducidos por la IL-4, de modo que los miRNAs se inducirían incrustados dentro de los tránscritos primarios de ambos. Puesto que la IL-4 juega un papel crucial en la evasión de la apoptosis y la resistencia a quimioterapia, la identificación de los genes y miRNAs regulados por la IL-4 contribuiría a un mayor esclarecimiento de los mecanismos moleculares de la señalización por la IL-4, lo que podría aprovecharse para buscar alternativas terapéuticas mediante la manipulación de esta vía, con inhibidores específicos o con nuevos abordajes más experimentales como el empleo de miRNAs.

7. ABSTRACT



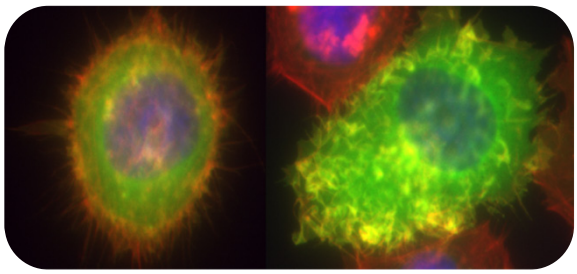
In previous studies, our group identified DOCK10 as an IL-4-induced gene in normal B cells (NBC) and chronic lymphocytic leukemias (CLL). Since it belongs to DOCK family proteins, a potential role as a regulator for Rho GTPases is suggested. In publication 3.1, we tried to answer some essential questions about DOCK10 function, such as: (1) its specificity to interact with 'classic' Rho GTPases, that we undertook by performing pulldown assays following transfection in 293T cell line; (2) its potential role as a GEF for 'classic' Rho GTPases, also by means of pulldown assays but in this case by creating a HeLa cell clone system with stable regulatable expression; and (3) its role in cell morphology, actin cytoskeleton dynamics, and membrane protrusions by fluorescence microscopy in the HeLa cell clone system. We found that the mutually exclusive first exon isoforms DOCK10.1 and DOCK10.2 interact specifically with nucleotide-free Cdc42 and Rac1 in vitro, and that DOCK10.1 induces an increase in the levels of activation of both GTPases. DOCK10.1 induces a transition from an elongated polygonal shape towards a more rounded, non-polygonal shape, with increase of filopodia and membrane ruffles. These changes are consistent with activation of both GTPases.

CLL pathogenesis is not well-known, and IL-4, as a B-cell growth factor, could play a role. IL-4 is anti-apoptotic in CLL, and several studies suggest that it could play an oncogenic role. Since IL-4 very likely exerts part of its effects by inducing changes in the gene expression profiles, and these have not been comprehensively studied in NBC and CLL, we performed a microarray study in both cell types (publication 3.2). We found that IL-4 regulates expression of at least 229 genes, 189 in CLL and 123 in NBC, being 89 common. Most of them are induced, and these suffer stronger changes. Changes are also more intense in CLL than in NBC, suggesting that the IL-4 pathway is very active in CLL. Most of the genes were not previously known as IL-4 targets. Moreover, we found that the gene response to IL-4 depends on basal expression of the prognostic marker ZAP-70, through a mechanism that involves NFκB transcription factor. We also identified genes that correlate with anti-apoptotic protection, such as HOMER2 and BCL6.

In publication 3.3, we studied, also by microarrays, the gene response of a type of non-conventional gene called miRNAs, which are important for regulation of protein expression. We identified 8 mature miRNAs, miR-21 (5p y 3p), miR-362 (3p y 5p), miR-500a-3p, miR-502-3p, y miR-532 (3p y 5p), that are induced by IL-4 in LLC. This occurs probably through a 'passive' mechanism, since miR-21 is intragenic to VMP1, the remaining miRNAs are intragenic to CLCN5, and both genes are induced by IL-4. Thus, the miRNAs would be induced embedded within VMP1 and CLCN5 primary transcripts. Because IL-4 play a crucial role in evasion from apoptosis and resistance to chemotherapy, identification of genes and miRNAs regulated by IL-4 could contribute to elucidate the full picture of signalling mechanisms triggered by IL-4. This

knowledge could be useful for searching new therapeutic alternatives through manipulation of this pathway with specific inhibitors or new experimental approaches such as the use of miRNAs.

8. BIBLIOGRAFÍA



Alcaraz-García MJ, Ruiz-Lafuente N, Sebastián-Ruiz S, Majado MJ, González-García C, Bernardo MV, Álvarez-López MR, Parrado A (2011). Human and mouse DOCK10 splicing isoforms with alternative first coding exon usage are differentially expressed in T and B lymphocytes. Hum Immunol 72: 531-537.

Aspenström P (1999). Effectors for the Rho GTPases. Curr Opin Cell Biol 11: 95-102.

Aspenström P, Fransson A, Saras J (2004). Rho GTPases have diverse effects on the organization of the actin filament system. Biochem J 377: 327-337.

Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP (2008). The impact of microRNAs on protein output. Nature 455: 64-71.

Baer C, Claus R, Frenzel LP, Zucknick M, Park YJ, Gu L, Weichenhan D, Fischer M, Pallasch CP, Herpel E, Rehli M, Byrd JC, Wendtner CM, Plass C (2012). Extensive promoter DNA hypermethylation and hypomethylation is associated with aberrant microRNA expression in chronic lymphocytic leukemia. Cancer Res 72: 3775-3785.

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.

Bhattacharya N, Reichenzeller M, Caudron-Herger M, Haebe S, Brady N, Diener S, Nothing M, Döhner H, Stilgenbauer S, Rippe K, Mertens D. (2015). Loss of cooperativity of secreted CD40L and increased dose-response to IL4 on CLL cell viability correlates with enhanced activation of NF-kB and STAT6. Int J Cancer 136: 65-73.

Bhoi S, Ljungström V, Baliakas P, Mattsson M1, Smedby KE, Juliusson G, Rosenquist R, Mansouri L (2016). Prognostic impact of epigenetic classification in chronic lymphocytic leukemia: the case of subset #2. Epigenetics 11: 449–455.

Bishop AL, Hall A (2000).Rho GTPase and their effector proteins. Biochem J 348: 241-255.

Bomben R, Gobessi S, Dal Bo M, Volinia S, Marconi D, Tissino E, Benedetti D, Zucchetto A, Rossi D, Gaidano G, Del Poeta G, Laurenti L, Efremov DG, Gattei V (2012). The miR-17~92 family regulates the response to Toll-like receptor 9 triggering of CLL cells with unmutated IGHV genes. Leukemia 26: 1584-1593.

Bueno MJ Pérez de Castro I, Malumbres M (2008). Control of cell proliferation pathways by microRNAs. Cell Cycle 7: 3143–3148.

Burger JA, Montserrat E (2013). Coming full circle: 70 years of chronic lymphocytic leukemia cell redistribution, from glucocorticoids to inhibitors of B-cell receptor signaling. Blood 121: 1501–1509.

Burgess M, Cheung C, Chambers L, Ravindranath K, Minhas G,Knop L, Mollee P, McMillan NA, Gill D (2012). CCL2 and CXCL2 enhance survival of primary chronic lymphocytic leukemia cells in vitro. Leuk Lymphoma 53: 1988-1998.

Buscaglia LE, Li Y (2011). Apoptosis and the target genes of microRNA-21. Chin J Cancer 30: 371-380.

Byrd JC, Gribben JG, Peterson BL, Grever MR, Lozanski G, Lucas DM, Lampson B, Larson RA, Caligiuri MA, Heerema NA (2006). Select high-risk genetic features predict earlier progression following

chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy. J Clin Oncol 24: 437–443.

Cahill N, Bergh AC, Kanduri M, Göransson-Kultima H, Mansouri L, Isaksson A, Ryan F, Smedby KE, Juliusson G, Sundström C, Rosén A, Rosenquist R (2013). 450K–array analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments. Leukemia 27: 150–158.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM (2002). Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 99: 15524–15529.

Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM (2005). A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353: 1793–1801.

Calpe E, Codony C, Baptista MJ, Abrisqueta P, Carpio C, Purroy N, Bosch F, Crespo M (2011). ZAP-70 enhances migration of malignant B lymphocytes toward CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation. Blood 118: 4401-4410.

Chaitidis P, O'Donnell V, Kuban RJ, Bermudez-Fajardo A, Ungethuem U, Kühn H (2005). Gene expression alterations of human peripheral blood monocytes induced by medium-term treatment with the TH2-cytokines interleukin-4 and -13. Cytokine 30: 366-377.

Chen L, Huynh L, Apgar J, Tang L, Rassenti L,Weiss A, Kipps TJ (2008). ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. Blood 111: 2685-2692.

Chen Z, Lund R, Aittokallio T, Kosonen M, Nevalainen O, Lahesmaa R (2003). Identification of novel IL-4/Stat6-regulated genes in T lymphocytes. J Immunol 171: 3627-3635.

Cherfils J, Chardin P (1999). GEFs: structural basis for their activation of small GTP-binding proteins. Trends Biochem Sci 24: 306-311.

Chhabra ES, Higgs HN (2007).The many faces of actin: matching assembly factors with cellular structures. Nat Cell Biol 9: 1110-1121.

Christensen LL, Tobiasen H, Holm A, Schepeler T, Ostenfeld MS, Thorsen K, Rasmussen MH, Birkenkamp-Demtroeder K, Sieber OM, Gibbs P, Lubinski J, Lamy P; COLOFOL steering group., Laurberg S, Oster B, Hansen KQ, Hagemann-Madsen R, Byskov K, Ørntoft TF, Andersen CL (2013). MiRNA-362-3p induces cell cycle arrest through targeting of E2F1, USF2 and PTPN1 and is associated with recurrence of colorectal cancer. Int J Cancer 133: 67-78.

Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci 102: 13944–13949.

Coscia M, Pantaleoni F, Riganti C, Vitale C, Rigoni M, Peola S, Castella B, Foglietta M, Griggio V, Drandi D, Ladetto M, Bosia A, Boccadoro M, Massaia M (2011). IGHV unmutated CLL B cells are more

prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. Leukemia 25: 828-837.

Cosimo E, McCaig AM, Carter-Brzezinski LJM, Wheadon H, Leach MT,Le Ster K, Berthou C, Durieu E, Oumata N, Galons H, Meijer L, Michie AM (2013). Inhibition of NF-kB-mediated signaling by the cyclin-dependent kinase inhibitor CR8 overcomes prosurvival stimuli to induce apoptosis in chronic lymphocytic leukemia cells. Clin Cancer Res 19: 2393-2405.

Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, Croce CM (2006). Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci USA 103: 7024–7029.

Côté JF, Vuori K (2002). Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. J Cell Sci 115: 4901–13.

Cuní S, Pérez-Aciego P, Pérez-Chacón G, Vargas JA, Sánchez A, Martín-Saavedra FM, Ballester S, García-Marco J, Jordá J, Durántez A (2004). A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. Leukemia 18: 1391–1400.

Czuchra A, Wu X, Meyer H, van Hengel J, Schroeder T, Geffers R, Rottner K, Brakebusch C (2005). Cdc42 is not essential for filopodium formation, directed migration, cell polarization, and mitosis in fibroblastoid cells. Mol Biol Cell 16: 4473-4484.

Damm F, Mylonas E, Cosson A, Yoshida K, Della Valle V, Mouly E, Diop M, Scourzic L, Shiraishi Y, Chiba K, Tanaka H, Miyano S, Kikushige Y, Davi F, Lambert J, Gautheret D, Merle-Béral H, Sutton L, Dessen P, Solary E, Akashi K, Vainchenker W, Mercher T, Droin N, Ogawa S, Nguyen-Khac F, Bernard OA (2014). Acquired initiating mutations in early hematopoietic cells of CLL patients. Cancer Discov 4: 1088–1101.

Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G, Sarfati M (1992). Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. J Exp Med 176: 1319–1326.

de Rooij MF, Kuil A, Geest CR, Eldering E, Chang BY, Buggy JJ, Pals ST, Spaargaren M (2012). The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. Blood 119: 2590–2594.

Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A (2007). Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest 117:112–121.

Del Giudice I, Chiaretti S, Tavolaro S, De Propris MS, Maggio R, Mancini F, Peragine N, Santangelo S, Marinelli M, Mauro FR, Guarini A, Foà R (2009). Spontaneous regression of chronic lymphocytic leukemia: clinical and biologic features of 9 cases. Blood 114: 638-646.

Del Pozo MA, Kiosses WB, Alderson NB, Meller N, Hahn KM, Schwartz MA (2002). Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. Nat Cell Biol 4: 232-239.

Di Leva G, Garofalo M, Croce CM (2014). MicroRNAs in cancer. Annu Rev Pathol 9: 287–314.

Diekmann D, Nobes CD, Burbelo PD, Abo A, Hall A (1995). Rac GTPase interacts with GAPs and target proteins through multiple effector sites. EMBO J 14: 5297-5305.

Dijkstra MK, van Lom K, Tielemans D, Elstrodt F, Langerak AW, van 't Veer MB, Jongen-Lavrencic M (2009). 17p13/TP53 deletion in B-CLL patients is associated with microRNA-34a downregulation. Leukemia 23: 625–627.

Dohner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L, Döhner K, Bentz M, Lichter P (2000). Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 343: 1910–1916.

Douglas RS, Capocasale RJ, Lamb RJ, Nowell PC, Moore JS (1997). Chronic lymphocytic leukemia B cells are resistant to the apoptotic effects of Transforming Growth Factor-β. Blood 89: 941-947.

Dürig J, Nückel H, Hüttmann A, Kruse E, Hölter T, Halfmeyer K, Führer A, Rudolph R, Kalhori N, Nusch A, Deaglio S, Malavasi F, Möröy T, Klein-Hitpass L, Dührsen U (2003). Expression of ribosomal and translation-associated genes is correlated with a favorable clinical course in chronic lymphocytic leukemia. Blood 101: 2748–2755.

Eichhorst B, Robak T2, Montserrat E, Ghia P, Hillmen P, Hallek M, Buske C; ESMO Guidelines Committee (2015). Chronic lymphocytic leukaemia: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 26 (Suppl. 5): v78–v84.

Esquela-Kerscher A, Slack FJ (2006). Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269.

Eulalio A, Huntzinger E, Izaurralde E (2008). Getting to the root of miRNA-mediated gene silencing. Cell 132: 9-14.

Fabbri G, Rasi S, Rossi D, Trifonov V, Khiabanian H, Ma J, Grunn A, Fangazio M, Capello D, Monti S, Cresta S, Famà R, De Paoli L, Bulian P, Gattei V, Guarini A, Deaglio S, Capello D, Rabadan R, Pasqualucci L, Dalla-Favera R, Foà R, Gaidano G (2011). Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. J Exp Med 208: 1389–1401.

Fabbri M, Bottoni A, Shimizu M, Spizzo R, Nicoloso MS, Rossi S, Barbarotto E, Cimmino A, Adair B, Wojcik SE, Valeri N, Calore F, Sampath D, Fanini F, Vannini I, Musuraca G, Dell'Aquila M, Alder H, Davuluri RV, Rassenti LZ, Negrini M, Nakamura T, Amadori D, Kay NE, Rai KR, Keating MJ, Kipps TJ, Calin GA, Croce CM (2011). Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia. JAMA 305: 59–67.

Fält S1, Merup M, Tobin G, Thunberg U, Gahrton G, Rosenquist R, Wennborg A (2005). Distinctive gene expression pattern in VH3-21 utilizing B-cell chronic lymphocytic leukemia. Blood 106: 681-689.

Feig LA (1999). Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. Nat Cell Biol 1: E25-27.

Ferracin M, Zagatti B, Rizzotto L, Cavazzini F, Veronese A, Ciccone M, Saccenti E, Lupini L, Grilli A, De Angeli C, Negrini M, Cuneo A (2010). MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia. Mol Cancer 9: 123.

Ferreira PG, Jares P, Rico D, Gómez-López G, Martínez-Trillos A, Villamor N, Ecker S, González-Pérez A, Knowles DG, Monlong J, Johnson R, Quesada V, Djebali S, Papasaikas P, López-Guerra M, Colomer D, Royo C, Cazorla M, Pinyol M, Clot G, Aymerich M, Rozman M, Kulis M, Tamborero D, Gouin A, Blanc J, Gut M, Gut I, Puente XS, Pisano DG, Martin-Subero JI, López-Bigas N, López-Guillermo A, Valencia A, López-Otín C, Campo E, Guigó R (2014). Transcriptome characterization by RNA sequencing identifies a major molecular and clinical subdivision in chronic lymphocytic leukemia. Genome Res 24: 212–226.

Fluge Ø, Bruland O, Akslen LA, Lillehaug JR, Varhaug JE (2006). Gene expression in poorly differentiated papillary thyroid carcinomas. Thyroid 16: 161–175.

Friedman DR, Weinberg JB, Barry WT, Goodman BK, Volkheimer AD, Bond KM, Chen Y, Jiang N, Moore JO, Gockerman JP, Diehl LF, Decastro CM, Potti A, Nevins JR (2009). A genomic approach to improve prognosis and predict therapeutic response in chronic lymphocytic leukemia. Clin Cancer Res 15: 6947-6955.

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

Fukui Y, Hashimoto O, Sanui T, Oono T, Koga H, Abe M, Inayoshi A, Noda M, Oike M, Shirai T, Sasazuki T (2001). Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature 412: 826-831.

Fulci V, Chiaretti S, Goldoni M, Azzalin G, Carucci N, Tavolaro S, Castellano L, Magrelli A, Citarella F, Messina M, Maggio R, Peragine N, Santangelo S, Mauro FR, Landgraf P, Tuschl T, Weir DB, Chien M, Russo JJ, Ju J, Sheridan R, Sander C, Zavolan M, Guarini A, Foà R, Macino G (2007). Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood 109: 4944-4951.

Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ (2000). Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol 164: 2200–2206.

Gadea G, Sanz-Moreno V, Self A, Godi A, Marshall CJ (2008). DOCK10-mediated Cdc42 activation is necessary for amoeboid invasion of melanoma cells. Curr Biol 18(19): 1456-1465.

Garrett MD, Self AJ, van Oers C, Hall A (1989). Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins. J Biol Chem 264: 10-13.

Gribben JG (2010). How I treat CLL up front. Blood 115(2): 187-97.

Hall A (1998).Rho GTPases and the actin cytoskeleton. Science 279: 509-514.

Hallek M, Wanders L, Ostwald M, Busch R, Senekowitsch R, Stern S, Schick HD, Kuhn-Hallek I, Emmerich B (1996). Serum β2-microglobulin and serum thymidine kinase are independent predictors of progression-free survival in chronic lymphocytic leukemia and immunocytoma. Leuk Lymphoma 22: 439–447.

Harada Y, Tanaka Y, Terasawa M, Pieczyk M, Habiro K, Katakai T, Hanawa-Suetsugu K, Kukimoto-Niino M, Nishizaki T, Shirouzu M, Duan X, Uruno T, Nishikimi A, Sanematsu F, Yokoyama S, Stein JV,

Kinashi T, Fukui Y (2012). DOCK8 is a Cdc42 activator critical for interstitial dendritic cell migration during immune responses Blood 119: 4451-4461.

Hayden EC (2008). Thousands of proteins affected by miRNA. Nature 454: 562.

Heasman SJ, Ridley AJ (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat Rev Mol Cell Biol 9: 690-701.

Hewamana S, Alghazal S, Lin TT, Clement M, Jenkins C,Guzman ML, Jordan CT, Neelakantan S, Crooks PA, Burnett AK, Pratt G, Fegan C, Rowntree C, Brennan P, Pepper C (2008). The NF-kappaB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. Blood 111: 4681-4689.

Hiramoto K, Negishi M, Katoh H (2006). Dock4 is regulated by RhoG and promotes Rac-dependent cell migration. Exp Cell Res 312: 4205-4216.

Hodge RG, Ridley AJ (2016). Regulating Rho GTPases and their regulators. Nat Rev Mol Cell Biol 8: 496-510.

Hoffman GR, Nassar N, Cerione RA (2000). Structure of the Rho family GTPbinding protein Cdc42 in complex with the multifunctional regulator RhoGDI. Cell 100: 345-356.

Huang GN, Huso DL, Bouyain S, Tu J, McCorkell KA,May MJ, Zhu Y, Lutz M, Collins S, Dehoff M, Kang S, Whartenby K, Powell J, Leahy D, Worley PF (2008). NFAT binding and regulation of T cell activation by the cytoplasmic scaffolding Homer proteins. Science 319: 476-481.

Humtsoe JO, Koya E, Pham E, Aramoto T, Zuo J, Ishikawa T, Kramer RH (2012). Transcriptional profiling identifies upregulated genes following induction of epithelial-mesenchymal transition in squamous carcinoma cells. Exp Cell Res 318: 379–390.

Hüttmann A, Klein-Hitpass L, Thomale J, Deenen R, Carpinteiro A, Nückel H, Ebeling P, Führer A, Edelmann J, Sellmann L, Dührsen U, Dürig J (2006). Gene expression signatures separate B-cell chronic lymphocytic leukaemia prognostic subgroups defined by ZAP-70 and CD38 expression status. Leukemia 20: 1774-1782.

Ikushima S, Inukai T, Inaba T, Nimer SD, Cleveland JL, Look AT (1997).Pivotal role for the NFIL3/E4BP4 transcription factor in interleukin 3-mediated survival of pro-B lymphocytes. Proc Natl Acad Sci USA 94: 2609-2614.

Jaffe AB, Hall A (2005). Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 21: 247-269.

Jantus Lewintre E, Reinoso Martín C, García Ballesteros C, Pendas J, Benet Campos C, Mayans Ferrer JR, García-Conde J (2009). BCL6: somatic mutations and expression in early-stage chronic lymphocytic leukemia. Leuk Lymphoma 50: 773-780.

Jelinek DF, Tschumper RC, Stolovitzky GA, Iturria SJ, Tu Y, Lepre J, Shah N, Kay NE (2003). Identification of a global gene expression signature of B-chronic lymphocytic leukemia. Mol Cancer Res 1: 346–361.

Joshi AD, Hegde GV, Dickinson JD, Mittal AK, Lynch JC, Eudy JD, Armitage JO, Bierman PJ, Bociek RG, Devetten MP, Vose JM, Joshi SS (2007). ATM, CTLA4, MNDA, and HEM1 in high versus low CD38 expressing B-cell chronic lymphocytic leukemia. Clin Cancer Res 13 (18 Pt 1): 5295-5304.

Kaminski A, Demaine A, Prentice A (1998). Cytoplasmic interleukin-4 (IL-4) and surface IL-4 receptor expression in patients with B-cell lymphocytic leukemia. Blood 92: 2188-2189.

Kanduri M, Cahill N, Göransson H, Enström C, Ryan F, Isaksson A, Rosenquist R (2010). Differential genome-wide array-based methylation profiles in prognostic subsets of chronic lymphocytic leukemia. Blood 115: 296-305.

Kim VN, Han J, Siomi MC (2009). Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10: 126-39.

Kipps TJ, Stevenson FK, Wu CJ, Croce CM, Packham G, Wierda WG, O'Brien S, Gribben J, Rai K (2017). Chronic lymphocytic leukaemia. Nat Rev Dis Primers 3: 16096.

Kiyokawa E, Hashimoto Y, Kobayashi S, Sugimura H, Kurata T, Matsuda M (1998). Activation of Rac1 by a Crk SH3-binding protein, DOCK180. Genes Dev 12: 3331-3336.

Klein Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, Freedman A, Inghirami G, Cro L, Baldini L, Neri A, Califano A, Dalla-Favera R (2001). Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med 194: 1625-1638.

Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, Ambesi-Impiombato A, Califano A, Migliazza A, Bhagat G, Dalla-Favera R (2010). The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell 17: 28–40.

Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, Freedman A, Inghirami G, Cro L, Baldini L, Neri A, Califano A, Dalla-Favera R (2001). Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med 194: 1625–1638.

Kulis M, Heath S, Bibikova M, Queirós AC, Navarro A, Clot G, Martínez-Trillos A, Castellano G, Brun-Heath I, Pinyol M, Barberán-Soler S, Papasaikas P, Jares P, Beà S, Rico D, Ecker S, Rubio M, Royo R, Ho V, Klotzle B, Hernández L, Conde L, López-Guerra M, Colomer D, Villamor N, Aymerich M, Rozman M, Bayes M, Gut M, Gelpí JL, Orozco M, Fan JB, Quesada V, Puente XS, Pisano DG, Valencia A, López-Guillermo A, Gut I, López-Otín C, Campo E, Martín-Subero JI (2012). Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. Nat Genet 44: 1236–1242.

Kulkarni K, Yang J, Zhang Z, Barford D (2011). Multiple factors confer specific Cdc42 and Rac protein activation by dedicator of cytokinesis (DOCK) nucleotide exchange factors. J Biol Chem 286: 25341-25351.

Kuramoto K, Negishi M, Katoh H (2009). Regulation of dendrite growth by the Cdc42 activator Zizimin1/Dock9 in hippocampal neurons. J Neurosci 87: 1794-1805.

Kwofie MA, Skowronski J (2008). Specific recognition of Rac2 and Cdc42 by DOCK2 and DOCK9 guanine nucleotide exchange factors. J Biol Chem 283: 3088-3096.

Lafarge ST, Johnston JB, Gibson SB, Marshall AJ (2014). Adhesion of ZAP-70+ chronic lymphocytic leukemia cells to stromal cells is enhanced by cytokines and blocked by inhibitors of the PI3-kinase pathway. Leuk Res 38: 109-115.

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001). Identification of novel genes coding for small expressed RNAs. Science 294: 853–858.

Landau DA, Clement K, Ziller MJ, Boyle P, Fan J, Gu H, Stevenson K, Sougnez C, Wang L, Li S, Kotliar D, Zhang W, Ghandi M, Garraway L, Fernandes SM, Livak KJ, Gabriel S, Gnirke A, Lander ES, Brown JR, Neuberg D, Kharchenko PV, Hacohen N, Getz G, Meissner A, Wu CJ (2014). Locally disordered methylation forms the basis of intratumor methylome variation in chronic lymphocytic leukemia. Cancer Cell 26: 813–825.

Lau NC, Lim LP, Weinstein EG, Bartel DP (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294: 858–862.

Lawrie CH, Ballabio E, Dyar OJ, Jones M, Ventura R, Chi J, Tramonti D, Gooding S, Boultwood J, Wainscoat JS, Hatton CS, Schuh A (2009). MicroRNA expression in chronic lymphocytic leukaemia. Br J Haematol 147: 398-402.

Le Bris Y, Struski S, Guièze R, Rouvellat C, Prade N, Troussard X, Tournilhac O, Béné MC, Delabesse E, Ysebaert L (2016). Major prognostic value of complex karyotype in addition to TP53 and IGHV mutational status in first-line chronic lymphocytic leukemia. Hematol Oncol 2016 Sep 28. doi: 10.1002/hon.2349

Lee RC, Ambros V (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science 294: 862–64.

Lee RC, Feinbaum RL, Ambros V (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 843–854.

Lee YW, Eum SY, Chen KC, Hennig B, Toborek M (2004). Gene expression profile in interleukin-4-stimulated human vascular endothelial cells. Mol Med 10: 19-27.

Lewis BP, Burge CB, Bartel DP (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20.

Li S, Moffett HF, Lu J, Werner L, Zhang H, Ritz J, Neuberg D, Wucherpfennig KW, Brown JR, Novina CD. (2011). MicroRNA expression profiling identifies activated B cell status in chronic lymphocytic leukemia cells. PLoS ONE 6: e16956.

Lin Q, Yang W, Baird D, Feng Q, Cerione RA (2006). Identification of a DOCK180-related guanine nucleotide-exchange factor that is capable of mediating a positive feedback activation of Cdc42. J Biol Chem 281: 35253-35262.

López-Guerra M, Roué G, Pérez-Galán P, Alonso R, Villamor N, Montserrat E, Campo E, Colomer D (2009). p65 activity and ZAP-70 status predict the sensitivity of chronic lymphocytic leukemia cells to the selective IkappaB kinase inhibitor BMS-345541. Clin Cancer Res 15: 2767-2776.

Lu TX, Munitz A, Rothenberg ME (2009). MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol 182: 4994-5002.

Lu X, Nechushtan H, Ding F, Rosado MF, Singal R, Alizadeh AA, Lossos IS (2005). Distinct IL-4-induced gene expression, proliferation, and intracellular signaling in germinal center B-cell-like and activated B-cell-like diffuse large-cell lymphomas. Blood 105: 2924–2932.

Lund R, Ahlfors H, Kainonen E, Lahesmaa AM, Dixon C, Lahesmaa R (2005). Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. Eur J Immunol 35: 3307-3319.

Lundin J, Kimby E, Bergmann L, Karakas T, Mellstedt H, Osterborg A (2001). Interleukin 4 therapy for patients with chronic lymphocytic leukaemia: a phase I/II study. Br J Haematol 112 (1):155-60.

Mainou-Fowler T, Proctor SJ, Miller S, Dickinson AM (2001). Expression and production of interleukin 4 in B-cell chronic lymphocytic leukaemia. Leuk Lymphoma 42: 689–698.

Marton S, Garcia MR, Robello C, Persson H, Trajtenberg F, Pritsch O, Rovira C, Naya H, Dighiero G, Cayota A (2008). Small RNAs analysis in CLL reveals a deregulation of miRNA expression and novel miRNA candidates of putative relevance in CLL pathogenesis. Leukemia 22: 330-338.

Medina PP, Nolde M, Slack FJ (2010). OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature 467: 86–90.

Meller N, Irani-Tehrani M, Ratnikov BI, Paschal BM, Schwartz MA (2004). The novel Cdc42 guanine nucleotide exchange factor, Zizimin1, dimerizes via the Cdc42-binding CZH2 domain. J Biol Chem 279: 37470–37476.

Meller N, Irani-Therani M, Kiosses WB, Del Pozo, Schwartz MA (2002). Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins. Nat Cell Biol 4: 639–647.

Meller N, Merlot S, Guda C (2005). CZH proteins: a new family of Rho-GEFs. J Cell Sci 118: 4937–4946.

Meller N, Westbrook MJ, Shannon JD, Guda C, Schwartz MA (2008). Function of the N-terminus of zizimin1: autoinhibition and membrane targeting. Biochem J 409: 525-533.

Messner B, Stütz AM, Albrecht B, Peiritsch S, Woisetschläger M (1997). Cooperation of binding sites for STAT6 and NF kappa B/rel in the IL-4-induced up-regulation of the human IgE germline promoter. J Immunol 159: 3330–3337.

Miyamoto Y, Yamauchi J, Sanbe A, Tanoue A (2007). Dock6, a Dock-C subfamily guanine nucleotide exchanger, has the dual specificity for Rac1 and Cdc42 and regulates neurite outgrowth. Exp Cell Res 313: 791-804.

Monypenny J, Zicha D, Higashida C, Oceguera-Yanez F, Narumiya S, Watanabe N (2009). Cdc42 and Rac family GTPases regulate mode and speed but not direction of primary fibroblast migration during platelet-derived growth factor-dependent chemotaxis. Mol Cell Biol 29: 2730–2747.

Moskalev EA, Luckert K, Vorobjev IA, Mastitsky SE, Gladkikh AA, Stephan A, Schrenk M, Kaplanov KD, Kalashnikova OB, Pötz O, Joos TO, Hoheisel JD (2012). Concurrent epigenetic silencing of wnt/β-catenin pathway inhibitor genes in B cell chronic lymphocytic leukaemia. BMC Cancer 12: 213.

Mraz M, Malinova K, Kotaskova J, Pavlova S, Tichy B, Malcikova J, Stano Kozubik K, Smardova J, Brychtova Y, Doubek M, Trbusek M, Mayer J, Pospisilova S (2009). MiR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. Leukemia 23: 1159–1163.

Namekata K, Enokido Y, Iwasawa K, Kimura H (2004). MOCA induces membrane spreading by activating Rac1. J Biol Chem 279: 14331-14337.

Negrini M, Cutrona G, Bassi C, Fabris S, Zagatti B,Colombo M, Ferracin M, D'Abundo L, Saccenti E, Matis S, Lionetti M, Agnelli L, Gentile M, Recchia AG, Bossio S, Reverberi D, Rigolin G, Calin GA, Sabbioni S, Russo G, Tassone P, Morabito F, Ferrarini M, Neri A (2014). MicroRNAome expression in chronic lymphocytic leukemia: comparison with normal B cell subsets and correlations with prognostic and clinical parameters. Clin Cancer Res 20: 4141-4153.

Nishihara H, Maeda M, Oda A, Tsuda M, Sawa H, Nagashima K, Tanaka S (2002).DOCK2 associates with CrkL and regulates Rac1 in human leukemia cell lines Blood 100: 3968-3974.

Nishikimi A, Meller N, Uekawa N, Isobe K, Schwartz MA, Maruyama M (2005). Zizimin2: a novel, DOCK180-related guanine nucleotide exchange factor expressed predominantly in lymphocytes. FEBS Lett 579: 1039-1046.

Nobes CD, Hall A (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81: 53–62.

Oakes CC, Claus R, Gu L, Assenov Y, Hüllein J, Zucknick M, Bieg M, Brocks D, Bogatyrova O, Schmidt CR, Rassenti L, Kipps TJ, Mertens D, Lichter P, Döhner H, Stilgenbauer S, Byrd JC, Zenz T, Plass C (2014). Evolution of DNA methylation is linked to genetic aberrations in chronic lymphocytic leukemia. Cancer Discov 4: 348–361.

Okada H, Banchereau J, Lotze MT (2003). Interleukin-4. In Thompson AW, Lotze MT, editors. The Cytokine Handbook, Vol I, London: Academic Press pp 227–262.

Pan X, Wang ZX, Wang R (2010). MicroRNA-21: a novel therapeutic target in human cancer. Cancer Biol Ther 10: 1224-1232.

Pangault C, Amé-Thomas P, Ruminy P, Rossille D, Caron G, Baia M, De Vos J, Roussel M, Monvoisin C, Lamy T, Tilly H, Gaulard P, Tarte K, Fest T (2010). Follicular lymphoma cell niche: identification of a preeminent IL-4-dependent T(FH)–B cell axis. Leukemia 24: 2080–2089.

Papakonstantinou N, Ntoufa S, Chartomatsidou E, Papadopoulos G, Hatzigeorgiou A, Anagnostopoulos A, Chlichlia K, Ghia P, Muzio M, Belessi C, Stamatopoulos K (2013). Differential microRNA profiles and their functional implications in different immunogenetic subsets of chronic lymphocytic leukemia. Mol Med 19: 115–123.

Paterson HF, Self AJ, Garrett MD, Just I, Aktories K, Hall A (1990). Microinjection of recombinant p21rho induces rapid changes in cell morphology. J Cell Biol 111: 1001-1007.

Pei L, Choi JH, Liu J, Lee EJ, McCarthy B, Wilson JM, Speir E, Awan F, Tae H, Arthur G, Schnabel JL, Taylor KH, Wang X, Xu D, Ding HF, Munn DH, Caldwell C, Shi H (2012). Genome-wide DNA methylation analysis reveals novel epigenetic changes in chronic lymphocytic leukemia. Epigenetics 7: 567–578.

Pellegrin S, Mellor H (2008). RHO GTPase Activation Assays. Curr Protoc Cell Biol. 2008 March; Chapter: Unit 14.8

Pickering BM, de Mel S, Lee M, Howell M, Habens F, Dallman CL, Neville LA, Potter KN, Mann J, Mann DA, Johnson PW, Stevenson FK, Packham G (2007).Pharmacological inhibitors of NF-kappaB accelerate apoptosis in chronic lymphocytic leukaemia cells. Oncogene 26: 1166-1177.

Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordóñez GR, Bignell GR, Ye K, Alipaz J, Bauer MJ, Beare D, Butler A, Carter RJ, Chen L, Cox AJ, Edkins S, Kokko-Gonzales PI, Gormley NA, Grocock RJ, Haudenschild CD, Hims MM, James T, Jia M, Kingsbury Z, Leroy C, Marshall J, Menzies A, Mudie LJ, Ning Z, Royce T, Schulz-Trieglaff OB, Spiridou A, Stebbings LA, Szajkowski L, Teague J, Williamson D, Chin L, Ross MT, Campbell PJ, Bentley DR, Futreal PA, Stratton MR (2010). A comprehensive catalogue of somatic mutations from a human cancer genome. Nature 463: 191–196.

Ponader S, Chen SS, Buggy JJ, Balakrishnan K, Gandhi V, Wierda WG, Keating MJ, O'Brien S, Chiorazzi N, Burger JA. (2012). The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. Blood 119: 1182–1189.

Porpaczy E, Bilban M, Heinze G, Gruber M, Vanura K, Schwarzinger I, Stilgenbauer S, Streubel B, Fonatsch C, Jaeger U (2009). Gene expression signature of chronic lymphocytic leukaemia with Trisomy 12. Eur J Clin Invest 39: 568-575.

Price LS, Leng J, Schwartz MA, Bokoch GM (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. Mol Biol Cell 9: 1863-1871.

Pruitt K, Der CJ (2001) Ras and Rho regulation of the cell cycle and oncogenesis. Cancer Lett 171: 1-10.

Puente XS, Beà S, Valdés-Mas R, Villamor N, Gutiérrez-Abril J, Martín-Subero JI, Munar M, Rubio-Pérez C, Jares P, Aymerich M, Baumann T, Beekman R, Belver L, Carrio A, Castellano G, Clot G, Colado E, Colomer D, Costa D, Delgado J, Enjuanes A, Estivill X, Ferrando AA, Gelpí JL, González B, González S, González M, Gut M, Hernández-Rivas JM, López-Guerra M, Martín-García D, Navarro A, Nicolás P, Orozco M, Payer ÁR, Pinyol M, Pisano DG, Puente DA, Queirós AC, Quesada V, Romeo-Casabona CM, Royo C, Royo R, Rozman M, Russiñol N, Salaverría I, Stamatopoulos K, Stunnenberg HG, Tamborero D, Terol MJ, Valencia A, López-Bigas N, Torrents D, Gut I, López-Guillermo A, López-Otín C, Campo E (2015). Non-coding recurrent mutations in chronic lymphocytic leukaemia. Nature 526: 519–524.

Puente XS, Pinyol M, Quesada V, Conde L, Ordóñez GR, Villamor N, Escaramis G, Jares P, Beà S, González-Díaz M, Bassaganyas L, Baumann T, Juan M, López-Guerra M, Colomer D, Tubío JM, López C, Navarro A, Tornador C, Aymerich M, Rozman M, Hernández JM, Puente DA, Freije JM, Velasco G, Gutiérrez-Fernández A, Costa D, Carrió A, Guijarro S, Enjuanes A, Hernández L, Yagüe J, Nicolás P, Romeo-Casabona CM, Himmelbauer H, Castillo E, Dohm JC, de Sanjosé S, Piris MA, de Alava E, San Miguel J, Royo R, Gelpí JL, Torrents D, Orozco M, Pisano DG, Valencia A, Guigó R, Bayés M, Heath S, Gut M, Klatt P, Marshall J, Raine K, Stebbings LA, Futreal PA, Stratton MR, Campbell PJ, Gut I, López-Guillermo A, Estivill X, Montserrat E, López-Otín C, Campo E (2011). Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature 475: 101-105.

Queiros AC, Villamor N, Clot G, Martinez-Trillos A, Kulis M, Navarro A, Penas EM, Jayne S, Majid A, Richter J, Bergmann AK, Kolarova J, Royo C, Russiñol N, Castellano G, Pinyol M, Bea S,

Salaverria I, López-Guerra M, Colomer D, Aymerich M, Rozman M, Delgado J, Giné E, González-Díaz M, Puente XS, Siebert R, Dyer MJ, López-Otín C, Rozman C, Campo E, López-Guillermo A, Martín-Subero JI (2015). A B-cell epigenetic signature defines three biologic subgroups of chronic lymphocytic leukemia with clinical impact. Leukemia 29: 598–605.

Quesada V, Conde L, Villamor N, Ordóñez GR, Jares P, Bassaganyas L, Ramsay AJ, Beà S, Pinyol M, Martínez-Trillos A, López-Guerra M, Colomer D, Navarro A, Baumann T, Aymerich M, Rozman M, Delgado J, Giné E, Hernández JM, González-Díaz M, Puente DA, Velasco G, Freije JM, Tubío JM, Royo R, Gelpí JL, Orozco M, Pisano DG, Zamora J, Vázquez M, Valencia A, Himmelbauer H, Bayés M, Heath S, Gut M, Gut I, Estivill X, López-Guillermo A, Puente XS, Campo E, López-Otín C (2012). Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. Nat Genet 44: 47–52.

Raveche ES, Salerno E, Scaglione BJ, Manohar V, Abbasi F, Lin YC, Fredrickson T, Landgraf P, Ramachandra S, Huppi K, Toro JR, Zenger VE, Metcalf RA, Marti GE (2007). Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. Blood 109: 5079–5086.

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403: 901–906.

Ribas J, Ni X, Castanares M, Liu MM, Esopi D, Yegnasubramanian S, Rodriguez R, Mendell JT, Lupold SE (2012). A novel source for miR-21 expression through the alternative polyadenylation of VMP1 gene transcripts. Nucleic Acids Res 40: 6821-6833.

Ridley AJ, Hall A (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70: 389-399.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70: 401-410.

Rodríguez A, Villuendas R, Yáñez L, Gómez ME, Díaz R, Pollán M, Hernández N, de la Cueva P, Marín MC, Swat A, Ruiz E, Cuadrado MA, Conde E, Lombardía L, Cifuentes F, Gonzalez M, García-Marco JA, Piris MA; Spanish National Cancer Centre (CNIO) (2007). Molecular heterogeneity in chronic lymphocytic leukemia is dependent on BCR signaling: clinical correlation. Leukemia 21: 1984-1991.

Rodríguez AE, Hernández JÁ, Benito R, Gutiérrez NC, García JL, Hernández-Sánchez M, Risueño A, Sarasquete ME, Fermiñán E, Fisac R, de Coca AG, Martín-Núñez G, de Las Heras N, Recio I, Gutiérrez O, De Las Rivas J, González M, Hernández-Rivas JM (2012). Molecular characterization of chronic lymphocytic leukemia patients with a high number of losses in 13q14. PLoS ONE 2012; 7 (11): e48485

Rong R, Ahn JY, Huang H, Nagata E, Kalman D Kapp JA, Tu J, Worley PF, Snyder SH, Ye K (2003). PI3 kinase enhancer-Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis. Nat Neurosci 6: 1153-1161.

Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, Yang L, Pickeral OK, Rassenti LZ, Powell J, Botstein D, Byrd JC, Grever MR, Cheson BD, Chiorazzi N, Wilson WH, Kipps TJ, Brown PO, Staudt LM (2001). Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 194: 1639–1647.

Rossi S, Shimizu M, Barbarotto E, Nicoloso MS, Dimitri F, Sampath D, Fabbri M, Lerner S, Barron LL, Rassenti LZ, Jiang L, Xiao L, Hu J, Secchiero P, Zauli G, Volinia S, Negrini M, Wierda W, Kipps TJ, Plunkett W, Coombes KR, Abruzzo LV, Keating MJ, Calin GA (2010). MicroRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. Blood 116: 945-952.

Rossman KL, Der CJ, Sondek J (2005). GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 6: 167–80.

Sandhu SK, Fassan M, Volinia S, Lovat F, Balatti V, Pekarsky Y, Croce CM (2013). B-cell malignancies in microRNA Eμ-miR-17~92 transgenic mice. Proc Natl Acad Sci USA 110: 18208–18213.

Santanam U, Zanesi N, Efanov A, Costinean S, Palamarchuk A, Hagan JP, Volinia S, Alder H, Rassenti L, Kipps T, Croce CM, Pekarsky Y (2010). Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. Proc Natl Acad Sci USA 107: 12210–12215.

Scheffzek K, Stephan I, Jensen ON, Illenberger D, Gierschik P (2000). The RacRhoGDI complex and the structural basis for the regulation of Rho proteins by RhoGDI. Nat Struct Biol 7: 122-126.

Schmidt A, Hall A (2002).Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 16: 1587-1609.

Schmitz AA, Govek EE, Böttner B, Van Aelst L (2000). Rho GTPases: signaling, migration, and invasion. Exp Cell Res 261: 1-12.

Schroder AJ, Pavlidis P, Arimura A, Capece D, Rothman PB (2002). Cutting edge: STAT6 serves as a positive and negative regulator of gene expression in IL-4-stimulated B lymphocytes. J Immunol 168: 996–1000.

Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Dürig J, Küppers R (2012). Cellular origin and pathophysiology of chronic lymphocytic leukemia. J Exp Med 209: 2183–2198.

Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N (2008). Widespread changes in protein synthesis induced by microRNAs. Nature 455: 58-63.

Shen CH, Stavnezer J (1998). Interaction of stat6 and NF-kappaB: direct association and synergistic activation of interleukin-4-induced transcription. Mol Cell Biol 18: 3395–3404.

Smonskey MT, Block AW, Deeb G, Chanan-Khan AA, Bernstein ZP, Miller KC, Wallace PK, Starostik P (2012). Monoallelic and biallelic deletions of 13q14.3 in chronic lymphocytic leukemia: FISH vs miRNA RT-qPCR detection. Am J Clin Pathol 137: 641–646.

Song F, Zheng H, Liu B, Wei S, Dai H, Zhang L, Calin GA, Hao X, Wei Q, Zhang W, Chen K (2009). An miR-502-binding site single-nucleotide polymorphism in the 3'-untranslated region of the SET8 gene is associated with early age of breast cancer onset. Clin Cancer Res 15: 6292-6300.

Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, Dayton BD, Ding H, Enschede SH, Fairbrother WJ, Huang DC, Hymowitz SG, Jin S, Khaw SL, Kovar PJ, Lam LT, Lee J, Maecker HL, Marsh KC, Mason KD, Mitten MJ, Nimmer PM, Oleksijew A, Park CH, Park CM, Phillips DC, Roberts AW, Sampath D, Seymour JF, Smith ML, Sullivan GM, Tahir SK, Tse C, Wendt MD, Xiao Y, Xue JC, Zhang H, Humerickhouse RA, Rosenberg SH, Elmore SW (2013). ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med 19: 202–208.

Stankovic T, Hubank M, Cronin D, Stewart GS, Fletcher D, Bignell CR, Alvi AJ, Austen B, Weston VJ, Fegan C, Byrd PJ, Moss PA, Taylor AM (2004). Microarray analysis reveals that TP53- and ATM-mutant B-CLLs share a defect in activating proapoptotic responses after DNA damage but are distinguished by major differences in activating prosurvival responses. Blood 103: 291–300.

Steele AJ, Prentice AG, Cwynarski K, Hoffbrand AV, Hart SM, Lowdell MW, Samuel ER, Wickremasinghe RG (2010). The JAK3-selective inhibitor PF-956980 reverses the resistance to cytotoxic agents induced by interleukin-4 treatment of chronic lymphocytic leukemia cells: potential for reversal of cytoprotection by the microenvironment. Blood 116: 4569–4577. .

Steffen A, Ladwein M, Dimchev GA, Hein A, Schwenkmezger L, Arens S, Ladwein KI, Margit Holleboom J, Schur F, Victor Small J, Schwarz J, Gerhard R, Faix J, Stradal TE, Brakebusch C, Rottner K (2013). Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. J Cell Sci 126: 4572–4588.

Stratowa C, Löffler G, Lichter P, Stilgenbauer S, Haberl P, Schweifer N, Döhner H, Wilgenbus KK (2001). CDNA microarray gene expression analysis of B-cell chronic lymphocytic leukemia proposes potential new prognostic markers involved in lymphocyte trafficking. Int J Cancer 91: 474–480.

Tanzer A, Stadler PF (2004). Molecular evolution of a microRNA cluster. J Mol Biol 339: 327–335.

Te Raa GD, Derks IA, Navrkalova V, Skowronska A, Moerland PD, van Laar J, Oldreive C, Monsuur H, Trbusek M, Malcikova J, Lodén M, Geisler CH, Hüllein J, Jethwa A, Zenz T, Pospisilova S, Stankovic T, van Oers MH, Kater AP, Eldering E (2015). The impact of SF3B1 mutations in CLL on the DNA-damage response. Leukemia 29: 1133–1142.

Thapa DR, Bhatia K, Bream JH, D'Souza G, Rinaldo CR, Wolinsky S, Detels R, Martínez-Maza O (2012). B-cell activation induced microRNA-21 is elevated in circulating B cells preceding the diagnosis of AIDS-related non-Hodgkin lymphomas. AIDS 26: 1177-1180.

Thieu VT, Nguyen ET, McCarthy BP, Bruns HA, Kapur R, Chang CH, Kaplan MH (2007). IL-4-stimulated NF-kappaB activity is required for Stat6 DNA binding. J Leukoc Biol 82: 370–379.

Thompson PA, O'Brien SM, Wierda WG, Ferrajoli A, Stingo F, Smith SC, Burger JA, Estrov Z, Jain N, Kantarjian HM, Keating MJ (2015). Complex karyotype is a stronger predictor than del(17p) for an inferior outcome in relapsed or refractory chronic lymphocytic leukemia patients treated with ibrutinibbased regimens. Cancer 121: 3612–3621.

Tybulewicz VL, Henderson RB (2009). Rho family GTPases and their regulators in lymphocytes. Nat Rev Immunol 9:630–44.

Valencia A, Chardin P, Wittinghofer A, Sander C (1991). The ras protein family: evolutionary tree and role of conserved amino acids. Biochemistry 30: 4637-4648.

Van Aelst L, D'Souza-Schorey C (1997). Rho GTPases and signaling networks. Genes Dev 11: 2295-2322.

Vargova K, Curik N, Burda P, Basova P, Kulvait V, Pospisil V, Savvulidi F, Kokavec J, Necas E, Berkova A, Obrtlikova P, Karban J, Mraz M, Pospisilova S, Mayer J, Trneny M, Zavadil J, Stopka T (2011). MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia. Blood 117: 3816-3825.

Vasudevan S, Tong Y, Steitz JA (2007). Switching from repression to activation: microRNAs can up-regulate translation. Science 318 (5858): 1931-1934.

Vetter IR, Wittinghofer A (2001). The guanine nucleotide-binding switch in three dimensions. Science 294: 1299-1304.

Vidali L, Chen F, Cicchetti G, Ohta Y, Kwiatkowski DJ (2006). Rac1-null mouse embryonic fibroblasts are motile and respond to platelet-derived growth factor. Mol Biol Cell 17: 2377-2390.

Vives V, Laurin M, Cres G, Larrousse P, Morichaud Z, Noel D, Côté JF, Blangy A (2011). The Rac1 exchange factor Dock5 is essential for bone resorption by osteoclasts. J Bone Miner Res 26: 1099-1110.

Wahlfors J, Hiltunen H, Heinonen K, Hämäläinen E, Alhonen L, Jänne J (1992). Genomic hypomethylation in human chronic lymphocytic leukemia. Blood 80: 2074–2080.

Wang J, Coombes KR, Highsmith WE, Keating MJ, Abruzzo LV (2004). Differences in gene expression between B-cell chronic lymphocytic leukemia and normal B cells: a meta-analysis of three microarray studies. Bioinformatics 20: 3166–3178.

Wang L, Lawrence MS, Wan Y, Stojanov P, Sougnez C, Stevenson K, Werner L, Sivachenko A, DeLuca DS, Zhang L, Zhang W, Vartanov AR, Fernandes SM, Goldstein NR, Folco EG, Cibulskis K, Tesar B, Sievers QL, Shefler E, Gabriel S, Hacohen N, Reed R, Meyerson M, Golub TR, Lander ES, Neuberg D, Brown JR, Getz G, Wu CJ (2011). SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. N Engl J Med 365: 2497–2506.

Wang L, Shalek AK, Lawrence M, Ding R, Gaublomme JT, Pochet N, Stojanov P, Sougnez C, Shukla SA, Stevenson KE, Zhang W, Wong J, Sievers QL, MacDonald BT, Vartanov AR, Goldstein NR, Neuberg D, He X, Lander E, Hacohen N, Regev A, Getz G, Brown JR, Park H, Wu CJ (2014). Somatic mutation as a mechanism of Wnt/beta-catenin pathway activation in CLL. Blood 124: 1089–1098.

Wang W, Li J, Zhu W, Gao C, Jiang R Li W, Hu Q, Zhang B (2014). MicroRNA-21 and the clinical outcomes of various carcinomas: a systematic review and meta-analysis. BMC Cancer 14: 819.

Watabe-Uchida M, John KA, Janas JA, Newey SE, Van Aelst L (2006). The Rac activator DOCK7 regulates neuronal polarity through local phosphorylation of stathmin/Op18. Neuron 51: 727-739.

Wells CM, Walmsley M, Ooi S, Tybulewicz V, Ridley AJ (2004). Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. J Cell Sci 117: 1259-1268.

Wennerberg K, Der CJ (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci 117: 1301-1312.

Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, Zhao H, Ibbotson RE, Orchard JA, Davis Z, Stetler-Stevenson M, Raffeld M, Arthur DC, Marti GE, Wilson WH, Hamblin TJ, Oscier DG, Staudt LM (2003). ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 101: 4944–4951.

Willimott S, Baou M, Naresh K, Wagner SD (2007). CD154 induces a switch in pro-survival Bcl-2 family members in chronic lymphocytic leukaemia. Br J Haematol 138: 721-732.

Wu X, Ramachandran S, Lin MC, Cerione RA, Erickson JW (2011). A minimal Rac activation domain in the unconventional guanine nucleotide exchange factor Dock180. Biochemistry50(6): 1070-80.

Xia JT, Chen LZ, Jian WH, Wang KB, Yang YZHe WL, He YL, Chen D, Li W (2014). MicroRNA-362 induces cell proliferation and apoptosis resistance in gastric cancer by activation of NF-κB signaling. J Transl Med 12: 33.

Yamamoto Y, Kosaka N, Tanaka M, Koizumi F, Kanai Y, Mizutani T, Murakami Y, Kuroda M, Miyajima A, Kato T, Ochiya T (2009). MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma. Biomarkers 14: 529-538.

Yang J, Zhang Z, Roe SM, Marshall CJ, Barford D (2009). Activation of Rho GTPases by DOCK exchange factors is mediated by a nucleotide sensor. Science 325: 1398–1402.

Yang L, Wang L, Zheng Y (2006). Gene targeting of Cdc42 and Cdc42GAP affirms the critical involvement of Cdc42 in filopodia induction, directed migration, and proliferation in primary mouse embryonic fibroblasts. Mol Biol Cell 17: 4675–4685.

Yelo E, Bernardo MV, Gimeno L, Alcaraz-García MJ, Majado MJ, Parrado A (2008). Dock10, a novel CZH protein selectively induced by interleukin-4 in human B lymphocytes. Mol Immunol 45: 3411-3418.

Zamorano J, Mora AL, Boothby M, Keegan AD (2001). NF-kappa B activation plays an important role in the IL-4-induced protection from apoptosis. Int Immunol 13: 1479–1487.

Zenz T, Mohr J, Eldering E, Kater AP, Bühler A, Kienle D, Winkler D, Dürig J, van Oers MH, Mertens D, Döhner H, Stilgenbauer S (2009). MiR-34a as part of the resistance network in chronic lymphocytic leukemia. Blood 113: 3801–3808.

Zhai H, Song B, Xu X, Zhu W, Ju J (2013). Inhibition of autophagy and tumor growth in colon cancer by miR-502. Oncogene 32: 1570-1579.

Zhang WJ, Li BH, Yang XZ, Li PD, Yuan Q, Liu XH, Xu SB, Zhang Y, Yuan J, Gerhard GS, Masker KK, Dong C, Koltun WA, Chorney MJ (2008). IL-4-induced Stat6 activities affect apoptosis and gene expression in breast cancer cells. Cytokine 42: 39-47.

Zheng Z, Venkatapathy S, Rao G, Harrington CA (2002). Expression profiling of B cell chronic lymphocytic leukemia suggests deficient CD1-mediated immunity, polarized cytokine response,

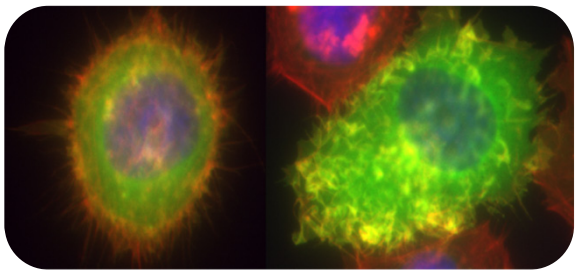
altered adhesion and increased intracellular protein transport and processing of leukemic cells. Leukemia 16: 2429-2437.

Zhou Y, Johnson JL, Cerione RA, Erickson JW (2013). Prenylation and membrane localization of Cdc42 are essential for activation by DOCK7. Biochemistry 52: 4354-4363.

Zhu DX, Zhu W, Fang C, Fan L, Zou ZJ, Wang YH, Liu P, Hong M, Miao KR, Liu P, Xu W, Li JY (2012). MiR-181a/b significantly enhances drug sensitivity in chronic lymphocytic leukemia cells via targeting multiple anti-apoptosis genes. Carcinogenesis 33: 1294-1301.

Ziller MJ, Gu H, Müller F, Donaghey J, Tsai LT, Kohlbacher O, De Jager PL, Rosen ED, Bennett DA, Bernstein BE, Gnirke A, Meissner A (2013). Charting a dynamic DNA methylation landscape of the human genome. Nature 500: 477–481.

9. PUBLICACIONES



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RESEARCH ARTICLE

Dock10, a Cdc42 and Rac1 GEF, induces loss of elongation, filopodia, and ruffles in cervical cancer epithelial HeLa cells

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ABSTRACT

Dock10 is one of the three members of the Dock-D family of Dock proteins, a class of guanine nucleotide exchange factors (GEFs) for Rho GTPases. Its homologs Dock9 and Dock11 are Cdc42 GEFs. Dock10 is required for maintenance of rounded morphology and amoeboid-type movement. Full-length isoforms of Dock10 have been recently cloned. Here, we address GTPase specificity and GEF activity of Dock10. In order of decreasing intensity, Dock10 interacted with nucleotide-free Rac1, Cdc42, and Rac3, and more weakly with Rac2, RhoF, and RhoG. Inducible expression of Dock10 in HeLa epithelial cells promoted GEF activity on Cdc42 and Rac1, and a morphologic change in two-dimensional culture consisting in loss of cell elongation, increase of filopodia, and ruffles. Area in contact with the substrate of cells that spread with non-elongated morphology was larger in cells expressing Dock10. Inducible expression of constitutively active mutants of Cdc42 and Rac1 in HeLa cells also induced loss of elongation. However, Cdc42 induced filopodia and contraction, and Rac1 induced membrane ruffles and flattening. When co-expressed with Dock10, Cdc42 potentiated filopodia, and Rac1 potentiated ruffles. These results suggest that Dock10 functions as a dual GEF for Cdc42 and Rac1, affecting cell morphology, spreading and actin cytoskeleton protrusions of adherent HeLa cells.

KEY WORDS: Dock10, Dock9, Dock11, Cdc42, Rac1, filopodia, membrane ruffles

INTRODUCTION

Rho GTPases are small proteins involved in actin cytoskeleton organization, cell shape, adhesion and movement (Wennerberg and Der, 2004; Aspenström et al., 2004; Heasman and Ridley, 2008). The "classic" Rho GTPases cycle between two forms, GDP- or GTP-bound. The GTP-bound is the "active" form, because it associates with downstream effectors. There are 12 genes encoding "classic" Rho GTPases in mammals, which can be grouped structurally and functionally in Cdc42-related (*Cdc42*, *RhoJ/TCL*, and *RhoQ/TC10*), Rac1-related (*Rac1*, *Rac2*, *Rac3*, and *RhoG*), RhoA-related (*RhoA*, *RhoB*, and *RhoC*), *RhoD*, and *RhoF/Rif*. Additional 8 genes encode the "atypical" Rho GTPases, constitutively bound to GTP. The activity of Rho GTPases is regulated by 3 classes of proteins: 1) Guanosine nucleotide exchange factors (GEFs), which stimulate the weak intrinsic exchange activity of Rho GTPases to promote the formation of the GTP-bound form; 2) GTPase-activating proteins (GAPs), which stimulate GTPase activity and conversion to the GDP-bound form; and 3) Rho GDP dissociation inhibitors (GDIs), which retain the GDP-bound form in the cytoplasm.

Dedicator of cytokinesis (Dock) proteins are large proteins which constitute a major class, together with the Dbl-homology proteins, of Rho GEFs (Rossman et al., 2005; Meller et al., 2005). Dock proteins are characterized by the presence of a carboxy-terminal domain known as CZH2, where their GEF function resides. There are 11 *Dock* genes in mammals, grouped in 4 families: A, B, C, and D. The D, or Zizimin, family, characterized by an N-terminal pleckstrin homology domain, is composed of 3 members, *Dock9/Zizimin1*, *Dock10/Zizimin3*, and *Dock11/Zizimin2*. Dock9 and Dock11, and their CZH2 domains, interact and activate Cdc42 (Meller et al., 2002; Nishikimi et al., 2005; Lin et al., 2006). Weak interactions of the CZH2 domain of Dock10 with Cdc42 and RhoJ have been reported (Nishikimi et al., 2005), but specificity of the complete Dock10 protein is unknown.

Rho GTPases play different roles in actin cytoskeleton dynamics. Cdc42-related, and RhoD and RhoF proteins, induce filopodia; Rac1-related proteins induce lamellipodia and membrane ruffles; RhoA-related proteins induce stress fibers (Wennerberg and Der, 2004; Aspenström et al., 2004; Chhabra and Higgs, 2007; Heasman and Ridley, 2008). When cultured on planar substrata without a migration stimulus, fibroblasts adhere and spread to adopt an elongated shape, and move randomly. Protrusive activity is determined by local regulation of Rho GTPase activation. Crosstalk regulation between GTPases favors their coordination. Thus, Cdc42 contributes to Rac1 activity (Nobes and Hall, 1995; Yang et al., 2006), Cdc42 and RhoG contribute to lamellipodia formation through Rac proteins (Monypenny et al., 2009), and Rac1 downregulates filopodia formation (Steffen et al., 2013).

We previously reported cloning of the full length coding sequences of the human and mouse *Dock10* genes (Yelo et al., 2008; Alcaraz-García et al., 2011). Two isoforms, designated Dock10.1 and Dock10.2, arise from alternative transcription start site usage. Expression of Dock10 is prominent in lymphoid organs, being T lymphocytes enriched in Dock10.1 and B lymphocytes in Dock10.2. Interleukin 4 upregulates Dock10 expression in B lymphocytes. Dock10 expression is also upregulated in aggressive cases of papillary thyroid carcinomas

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(Fluge et al., 2006), and in the epithelial to mesenchymal transition of squamous carcinoma cells (Humtsoe et al., 2012). What we know about the role of Dock10 comes from a single study using gene silencing, showing Dock10 as a factor that sustains the rounded morphology and amoeboid-type movement in melanoma cells (Gadea et al., 2008).

In this paper, we aimed to investigate Dock10 function by defining, for the first time, the specificity of the complete Dock10 protein for "classic" Rho GTPases, and studying its effects in human HeLa cells, using stable inducible expression. Our results show that Dock10 interacts with and activates Cdc42 and Rac1. Dock10 promotes a morphological transition from polygonal elongated to more rounded, non-polygonal cells. These cells develop abundant filopodia, frequently spread their area in contact with the substrate while retaining the non-elongated shape, and had increased ruffling activity. These results suggest that Dock10 is a GEF with broader specificity than its zizimin homologs, targeting Cdc42 but also Rac proteins.

MATERIALS AND METHODS

Cell lines

Human embryonal kidney (HEK) 293T cells, monkey kidney COS-1 cells, and human cervix carcinoma epithelial HeLa cells, were cultured on plastic flasks in Dulbecco's minimum essential medium supplemented with 10% Fetal Calf Serum (FCS; Biowhittaker, Cambrex, East Rutherford, NJ), 50 U/ml penicillin, 50 U/ml streptomycin, 2.5 µg/ml amphotericin B, and 2 mM L-glutamine ("complete medium", CM) at 37℃ in a humid atmosphere of 5% CO_2. The three cell lines grow as monolayers with fibroblast-like morphology, and were maintained subconfluent by detachment with trypsin 0.05%-EDTA 0.02% in PBS (EuroClone, Milan, Italy) and routine subculture.

In vitro interaction assays

GTPases binding assays were performed by GST pull-down experiments. *E. coli* BL21 DE3 cells transformed with plasmid constructs for inducible expression of N-terminally GST bound Cdc42, Rac1, Rac2 (generated from plasmid published in Hoppe and Swanson, 2004), Rac3 (generated from plasmid published in Hajdo-Milasinović et al., 2007), RhoA, RhoD (generated from plasmid published in Roberts et al., 2008), RhoF-SAAX (generated from a plasmid given by H. Mellor, University of Bristol, UK), RhoG-SAAX, RhoJ, and RhoQ (Neudauer et al., 1998) proteins, or GST alone, were grown in LB medium with 125 µg/ml of ampicillin and treated with 0.5 mM IPTG for 3 h. The plasmids used in this study, and the procedures to generate them, are listed in supplementary material Table S1. HEK 293T cells were transfected for 24 h with plasmid constructs for transient expression of FLAG-Dock9 (Meller et al., 2004), Dock10.1, HA-Dock10.1, Dock10.2, HA-Dock10.2 (generated from plasmids published in Alcaraz-García et al., 2011), Dock11, and HA-Dock11 (generated from plasmid published in Lin et al., 2006), using lipofectamine reagent (Invitrogen), following the manufacturer's instructions. Bacterial pellets were resuspended in Lysis Buffer A containing 50 mM Tris·Cl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, proteinase inhibitor cocktail cOmplete, EDTA free (Roche, Basel, Switzerland), and 100 mg/ml lysozyme, and sonicated. Bacterial lysates were cleared by centrifugation and bound for 1 h on glutathione-sepharose 4B beads (GE Healthcare, Little Chalfont, UK). Beads were then washed in Tris Wash Buffer A containing 50 mM Tris·Cl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% Triton X-100, and cOmplete EDTA free, and preserved at −80℃ in Tris Wash Buffer A with 10% glycerol. Protein loading in beads was quantified in Coomassie stained SDS-PAGE gels using BSA standards. A volume of beads containing 200 µg of loaded recombinant protein was washed three times and resuspended in 1 ml of either Solution A [20 mM Tris·Cl pH 7.5, 50 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.1% Triton X-100, cOmplete EDTA free, and 5% glycerol (nucleotide-depleted)], Solution B [same as Solution A but with 1 mM GDP (Sigma-Aldrich, St-Louis, MI) and 10 mM $MgCl_2$ instead of EDTA (GDP-loaded)], or Solution C [same as Solution B but with 1 mM GTP (Sigma-Aldrich) instead of GDP (GTP-loaded)]. Total protein extracts of transfected 293T cells (200 µl) were added to beads and incubated with end-over-end shaking at 4℃ overnight. Beads were then washed again, and SDS loading buffer was added. Proteins were denatured for 10 min at 100℃, loaded onto SDS-PAGE gels and immunoblotted using HA, FLAG, or Dock specific antibodies. Antibodies used in this work and their dilutions for different uses are listed in supplementary material Table S2.

Generation of stable cell clones with regulatable Dock10 expression

Stable clones with regulatable HA-Dock10 expression of HeLa cells were generated using the tet-off system following a procedure previously described with modifications (Parrado et al., 2000). HeLa-tTA cell clones were generated by transfection with the pUHD-15-1-Puro plasmid (Bernardo et al., 2007), using lipofectamine, and selected in 150 mm Falcon Tissue Culture-treated dishes (Corning Inc., Corning, NY) using CM supplemented with 1 µg/ml puromycin during 2–3 weeks. Colonies were gently scratched and aspired using micropipette points, and reseeded in 96-well plates filled with CM supplemented with puromycin. Isolated colonies were grown and regulation of transactivation by doxycycline (dox) was checked using the reporter plasmid pUHC-13-3, which drives expression of luciferase under the control of a promoter inducible by tTA (Gossen and Bujard, 1992). Dox binds and inactivates the tTA. The clone that best regulated transactivation by dox, designated HeLa-tTA, was subsequently transfected with the pJAG4-HA-Dock10 plasmid, which drives expression of HA-tagged Dock10 under the tTA-inducible promoter (generated by PCR cloning of Dock10 into pJAG4, a modified version of pJEF4; Parrado et al., 2000). HeLa cell clones were isolated during 2–3 weeks in CM with 1 µg/ml puromycin, 0.5 mg/ml G418, and 2 ng/ml dox, and colonies were grown following the same procedure as above with the same media used for selection. Colonies were checked for HA-Dock10 expression by western blot analysis, using extracts from replicate aliquots of cells washed free of dox and reseeded in CM either containing 2 ng/ml dox or lacking dox. One of the positive clones, C33, followed a third round of transfection either with pJAG2-EGFP-Cdc42Q61L or with pJAG2-EGFP-Rac1Q61L (generated from plasmids published in Subauste et al., 2000), which drive expression of the EGFP-tagged Cdc42 or Rac1 constitutively active mutants under the tTA inducible promoter. HeLa cell clones were isolated during 2–3 weeks in CM with 1 µg/ml puromycin, 0.5 mg/ml G418, 10 µg/ml zeocin and 2 ng/ml dox. The HeLa-tTA subline was subjected to a second round of transfection with constitutively active mutant GTPase plasmids and selection with puromycin, zeocin and dox. Single clones expressing EGFP-bound Cdc42 or Rac1 and double clones expressing HA-Dock10.1 and the EGFP-bound Cdc42 or Rac1 were identified after washing the cells free of dox in the selection dish, and culturing for 24 h in the absence of dox. Positive colonies were detected by green fluorescence using a JuLI™ Smart Fluorescence Cell Analyzer (NanoEnTek Inc, Seoul, Korea), then collected and grown following the same procedure as above with the same media used for selection. Colonies were grown and checked for expression of the GTPases western blot analysis as explained above.

Cdc42/Rac activation assays

When bound to GTP, Rho GTPases interact with their effectors (Aspenström, 1999). PAK1 interacts with Cdc42·GTP and Rac1·GTP through its p21 binding domain (PBD). Replicate aliquots of the HeLa clones with regulatable expression of HA-Dock10 cultured for 24 h in the presence and absence of dox were assayed by pull down using GST-PAK1-PBD bound beads. Cell were lysed in Lysis Buffer B containing 50 mM Tris·Cl pH 7.2, 0.5 M NaCl, 10 mM $MgCl_2$, 1% Triton X-100, and cOmplete EDTA free. GST-PAK1-PBD beads (40 µl) were added to cleared lysates (500 µl) and incubated with end-over-end shaking at 4℃ for 1 h. Beads were then washed in Tris Wash Buffer B containing 50 mM Tris-Cl pH 7.2, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100, and cOmplete EDTA free, and SDS loading buffer was added. Proteins were

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denatured for 10 min at 100℃, loaded onto SDS-PAGE gels and immunoblotted using Cdc42 or Rac1 specific antibodies. In different control experiments, HeLa cells were treated with 100 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), or HeLa protein extracts were incubated either with 1 mM GDP or 100 µM GTPγS (Sigma-Aldrich), a non hydrolyzable GTP analog.

Western blot analysis

Proteins fractionated in SDS PAGE gels were electroblotted onto nitrocellulose membranes. Blots were blocked in TBST with 5% Difco™ skim milk (BD) for 1 h, then incubated with primary antibodies at the dilutions indicated in supplementary material Table S2 in TBST with 0.5% skim milk for 2 h. Following three washes of 5 min each in TBST, membranes were incubated with secondary antibodies in TBST with 2.5% skim milk for 1 h. After four final washes of 10 min each in TBST, immunoreactive proteins were detected using the Amersham ECL or ECL Plus Western Blotting Detection Reagents (GE Healthcare). Chemiluminescence images were acquired and quantitated in a Molecular Imager ChemiDoc™ XRS+ with Image Lab software (Bio-Rad Laboratories, Hercules, CA).

Microscopy

HeLa cells were grown on to BioCoat collagen-coated chamber glass slides (Corning Inc.) and on to 12 mm BioCoat Poly-L-lysine-coated coverslips (Corning Inc.). Preparations were labelled using the F-actin visualization biochem kit (Cytoskeleton Inc., Denver, CO), following the manufacturer's instructions with minor modifications which consisted in the inclusion of incubation with anti-HA antibody for 1 h at 4℃ after the permeabilization step, followed by three washes, and incubation with anti-rat-Alexa Fluor 488, 100 nM phalloidin-rhodamine (TRITC), and 1 µg/ml DAPI before final washing steps. Dako fluorescent mounting medium was placed between slides and coverslips. Cells were examined in an Eclipse T*i* inverted microscope (Nikon Instruments Inc., Melville, NY). Fluorescence images were acquired and analyzed using the NIS Elements software, including measures of cell area. Cell counting and classification in 4 subsets (polygonal elongated, non-polygonal/non-elongated, unclear/early spreading, and small round) were performed manually on F-actin labelled preparations. HeLa cells typically spread longitudinally and adopt a polygonal shape, with thick F-actin fibers delineating the cell edges. Some polygonal cells not having a strictly elongated appearance were also included in the group. The non-polygonal/non-elongated subset included cells that spread with a more rounded shape and lack stress fibers. Round cells included the mitotic, early post-mitotic or apoptotic cells, small and spherical. Last, the transitional group consisted of non-spherical cells with little spreading. Phase contrast and green fluorescent time lapse images of cells grown on to poly-L-lysine-coated coverslips were acquired in the Nikon Eclipse microscope. Coverslips were mounted in a heated stage circular chamber and cultured in buffer containing 10 mM HEPES, 147 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 13 mM D-glutamine.

RESULTS

Interaction of Dock10 with Rho GTPases

To determine the specificity of Dock10 for "classic" Rho GTPases, *in vitro* interaction assays were performed. Total protein extracts of 293T cells transfected with HA-Dock10.1, HA-Dock10.2, HA-Dock11, and FLAG-Dock9 were assayed for precipitation by GST-bound Cdc42, Rac1, Rac2, Rac3, RhoA, RhoD, RhoF, RhoG, RhoJ (TCL), and RhoQ (TC10). Using nucleotide-free forms of the GTPases, we found that both Dock10 isoforms interacted, in order of decreasing intensity, with Rac1, Cdc42, and Rac3, and weakly with Rac2, RhoF, and RhoG; Dock11 interacted with Cdc42, and weakly with Rac1, and RhoJ; and Dock9 interacted with Cdc42, and weakly with Rac1, and RhoD (Fig. 1A). The Zizimin proteins did not interact with GDP- or GTP-loaded Cdc42 or Rac1 (Fig. 1B). Similar results were obtained with non HA-tagged Dock10.1 and

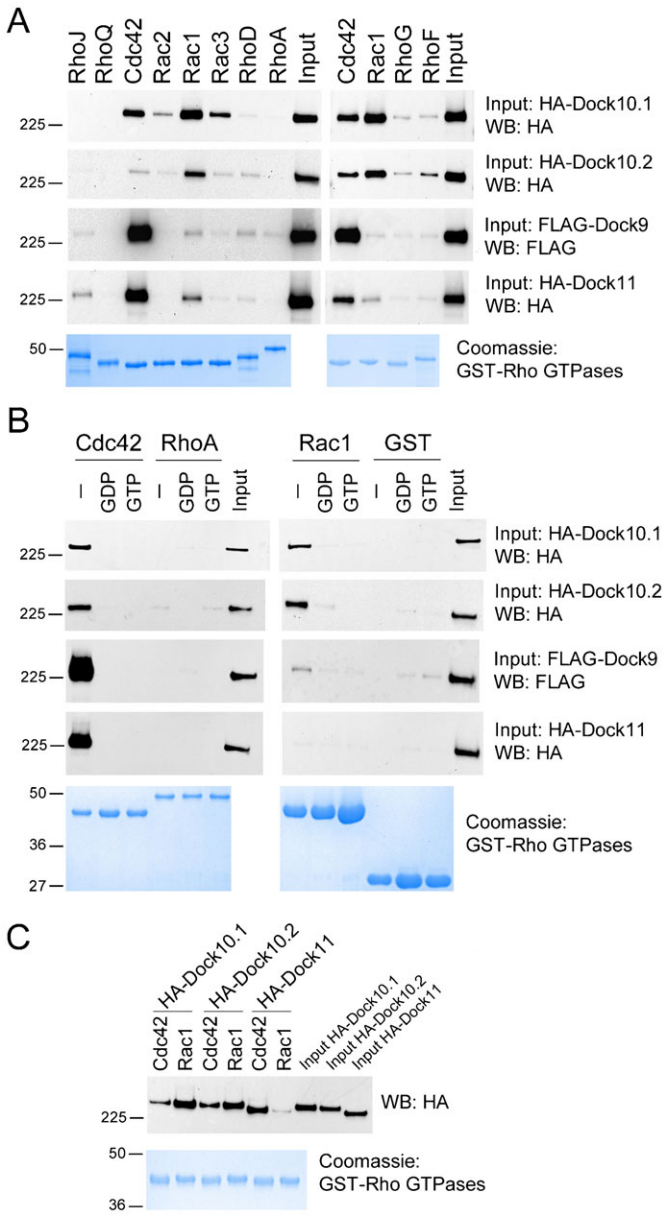

Fig. 1. *In vitro* interactions of Zizimin proteins with Rho GTPases. (A) Interactions of Zizimin proteins, expressed in 293T cells, with the nucleotide-free forms of the "classic" Rho GTPases. GST pull-down assays using total protein extracts from 293T cells transfected with FLAG-Dock9, HA-Dock10.1, HA-Dock10.2, and HA-Dock11 and recombinant GST-bound Cdc42, Rac1, Rac2, Rac3, RhoA, RhoD, RhoF, RhoG, RhoJ, and RhoQ proteins. (B) As in A, but using the nucleotide-free forms, and the GDP- or GTP-loaded forms of GST-bound Cdc42, Rac1, and RhoA, and GST alone. (C) As in A, but loading only the HA-tagged proteins, HA-Dock10.1, HA-Dock10.2, and HA-Dock11, precipitated by the nucleotide-free forms of the GST-bound Cdc42 and Rac1. The position of the size markers are indicated in kDa to the left.

Dock10.2 (data not shown). Both Dock10 isoforms interacted with Rac1 with similar intensity, equivalent to that of Dock11 with Cdc42 (Fig. 1C). Interactions of both Dock10 isoforms with Cdc42, though slightly less intense than those with Rac1, were still much higher than those of Dock9 and Dock11 with Rac1. Therefore, results of our interaction assays are consistent with specificity of Dock9 and Dock11 for Cdc42, and dual specificity of Dock10 for Rac1 and Cdc42.

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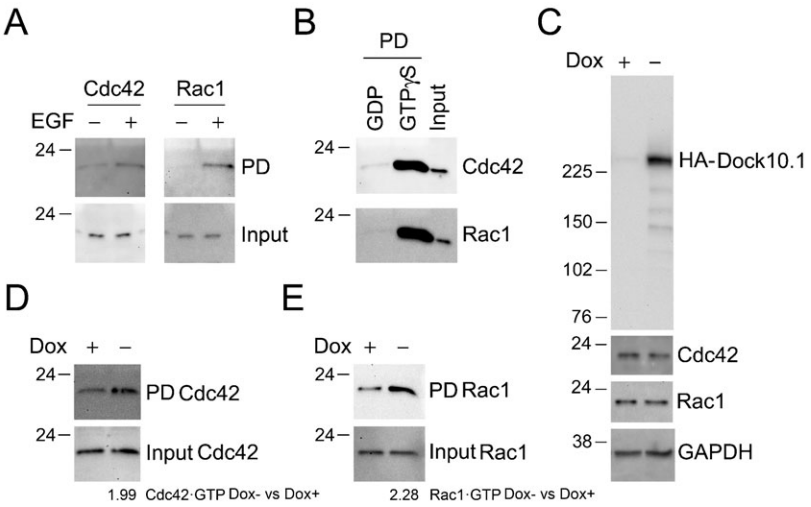

Fig. 2. Activation of Cdc42 and Rac1 by Dock10.1. (A) Activation of Cdc42 and Rac1 induced by treatment with 100 ng of EGF per ml for 3 min in HeLa cells by GST-PAK1-PBD pulldown assays. (B) Activation of Cdc42 and Rac1 using GDP (negative control) and GTPγS treated HeLa extracts, by GST-PAK1-PBD pulldown assays. (C) Inducible expression of HA-Dock10.1 in a HeLa cell clone did not change the levels of expression of Cdc42 and Rac1. Total protein extracts were performed 24 h after washing the cells free of dox and reseeding with or without 2 ng/ml dox. (D,E) Activation of Cdc42 (D) and Rac1 (E) in the HeLa clone expressing HA-Dock10.1 by GST-PAK1-PBD pulldown assays using protein extracts obtained as in C. Numbers underneath the blots represent ratios between the Dox− and Dox+ signals, normalized by input, from pulled down GTPases·GTP. Inputs are depicted as loading controls for the assays in A,B,D,E. The position of the size markers are indicated in kDa to the left. PD, pulldown. Dox, doxycycline.

Activation of Rho GTPases by Dock10.1

The ability of interacting *in vitro* with the nucleotide-free form, but not with the nucleotide-loaded forms of small GTPases, typifies the stages in GEF–small-GTPase interactions, where the GEFs stabilize the nucleotide-free form of the GTPase until GTP then displaces the GEF. To test the suggested role for Dock10 as a GEF for Cdc42 and Rac1, *in vitro* activation assays were performed by pulldown with GST-PAK1-PDB. Previously, we set up controls for this assay, such as a known model of Cdc42 and Rac1 activation by EGF in HeLa cells (Kurokawa et al., 2004 (Fig. 2A), and GDP-loaded, and GTPγS-loaded HeLa protein extracts (Fig. 2B). However, significant results were not obtained using the transient transfection expression vectors in 293T cells, COS-1 cells, or HeLa cells (data not shown), likely because insufficient transfection efficiency. To circumvent this problem, stable clones of the HeLa cell line with inducible expression of HA-Dock10.1 were generated. Three out of 28 clones expressed high levels of Dock10.1 following dox withdrawal with the expected size of 250K. Clone C33 was selected for further study. Tight repression by dox was checked for C33 (Fig. 2C). First, it was checked that expression of Dock10 did not significantly affect the levels of expression of Cdc42 or Rac1. Then, it was shown that increased amounts of Cdc42 and Rac1 were precipitated by GST-PAK1-PBD from protein extracts of clone C33 following 24 h of dox withdrawal compared with control cultures in the presence of dox, indicating that expression of Dock10.1 induces activation of Cdc42 and Rac1 (Fig. 2D and Fig. 2E, respectively). Therefore, both *in vitro* interaction and activation assays suggest that Dock10 acts as a GEF for Rac1 and Cdc42.

Effects of Dock10.1 in cell morphology and on actin cytoskeleton

Because small GTPases play essential roles in actin cytoskeleton dynamics, we studied the effects of Dock10 expression in cell

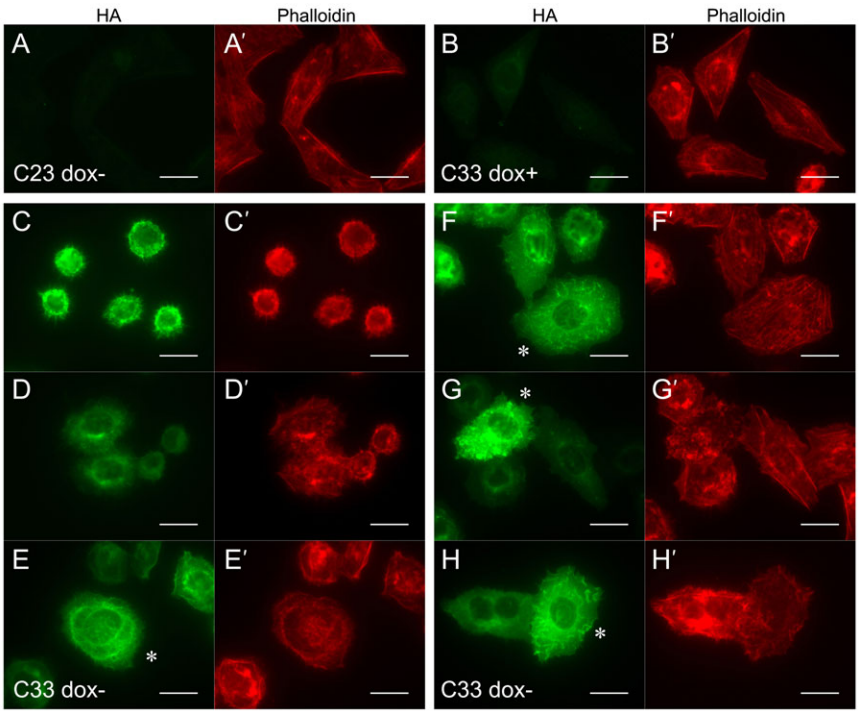

Fig. 3. Induction of loss of cell elongation, filopodia and ruffles by Dock10.1 expression and colocalization of Dock10 with filopodia and ruffles. Immunofluorescence microscopy analysis of the negative control clone C23 cultured for 24 h free of dox, labelled with HA-FITC (A), and phalloidin-TRITC (A′), and the inducible HA-Dock10.1 HeLa clone C33 cultured for 24 h after washing the cells free of dox and reseeding with (B,B′) or without 2 ng/ml dox (C–H′) onto collagen-coated chamber slides. Polygonal elongated cells exhibit stress fibers, particularly thick at the cell edges (A′,B′). Micrographs C–D′ (all the cells displayed) and E,E′ (cell labelled with an asterisk) illustrate the highly frequent presence of abundant filopodia in HA-Dock10.1 expressor cells that have lost the normal elongated morphology of HeLa cells, and the different levels of spreading found. Micrographs F–H′ (cells labelled with an asterisk) illustrate the presence of ruffles in non-polygonal spread cells. Dock10 colocalizes with filopodia and ruffles but not with stress fibers, as exemplified in the rare non-polygonal cell with ruffles, still displaying stress fibers, shown in F,F′. Objective magnification, 60×. Dox, doxycycline. Scale bars, 25 μm.

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morphology and on actin cytoskeleton. First, we examined a negative control clone, C23, and the positive clone C33 by fluorescence microscopy following double labelling with FITC-conjugated HA antibody and TRITC-conjugated phalloidin. After washing free of dox and seeding the C33 cells onto collagen-coated glass, cells attached to the surface and spread with non-polygonal, more rounded shape compared to most of the control clone C23 minus or plus dox, or the C33 cells in the presence of dox, which spread with polygonal elongated shape. In the elongated morphology, F-actin was arranged in parallel stress fibers in the direction of elongation and delineating the cell edges with polygonal shape (Fig. 3A–B′). Normal elongated HeLa cells exhibit dynamic protrusive membrane activity at the ends of the longitudinal axis of the cell, and occasional activity at the cell sides, as assessed by time lapse phase contrast analysis of cells cultured on poly-L-lysine coverslips (supplementary material Movie 1). Most non-polygonal cells from clone C33 induced to express HA-Dock10.1 were rich in filopodia (Fig. 3C–E′), and frequently developed membrane ruffles (Fig. 3F–H′; supplementary material Movie 2). Dock10.1 colocalized with F-actin in filopodia and membrane ruffles, but not in stress fibers (Fig. 3F,F′, showing a rare non-polygonal cell expressing Dock10.1 which exhibits ruffles and stress fibers). Induction of filopodia and ruffles are consistent with the proposed role of Dock10 as a GEF for Cdc42 and Rac1. These initial findings suggest that expression of Dock10.1 in HeLa cells induces loss of cell elongation.

Effects of Dock10.1 and the Cdc42Q61L and Rac1Q61L constitutively active mutants in cell morphology

Cdc42 and Rac1 may affect actin cytoskeleton organization in different ways depending on their interactions with GEFs expressed by cells. To relate the effects produced by Dock10.1 with activation of one or another GTPase, Cdc42Q61L and Rac1Q61L mutants N-terminally fused to EGFP transfectants were generated. The small GTPase Q61L mutants are constitutively active because their inability to hydrolyze bound GTP (Rossman et al., 2005). Single inducible clones expressing the GTPases, and double clones expressing the GTPases and HA-Dock10.1, were isolated. Positive clones were detected by fluorescence, and inducible expression of the EGFP-bound GTPase mutant proteins, and of HA-Dock10.1 in clones derived from C33, was confirmed by western blot analysis (Fig. 4).

The EGFP-GTPase clones were examined in parallel with the parental HeLa clone, the control clone C23, and the HA-Dock10 clone C33 cultured on poly-L-lysine-coated glass by fluorescence microscopy following labelling with phalloidin. Single clones expressing the GTPases also had less polygonal elongated cells following dox withdrawal (Fig. 5). As a rule, double clones co-expressing the GTPase mutants and the Dock10 protein,

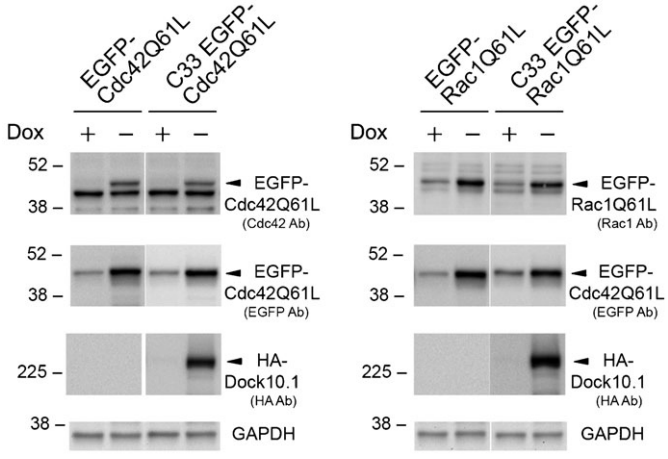

Fig. 4. Generation of HeLa cell clones expressing EGFP-coupled, constitutively active Cdc42Q61L and Rac1Q61L mutants. Western blot analyses of the inducible EGFP-Cdc42Q61L and EGFP-Rac1Q61L mutants generated from HeLa-tTA clone and double inducible HA-Dock10.1 and EGFP-GTPaseQ61L mutants generated from HeLa clone C33. Protein extracts were performed from cells cultured in the presence and absence of 2 ng/ml dox for 24 h. The EGFP-GTPase proteins were detected with Cdc42 or Rac1 antibodies and with EGFP antibody. The position of the size markers are indicated in kDa to the left. Dox, doxycycline.

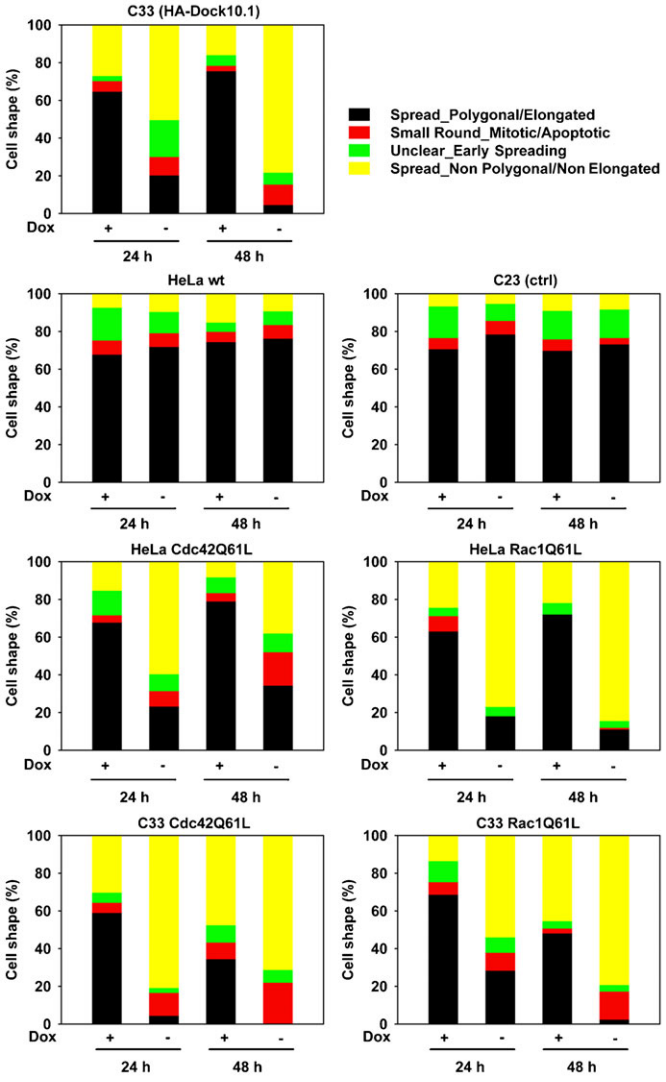

Fig. 5. Cell morphology changes induced by expression of HA-Dock10.1 and constitutively active EGFP-Cdc42Q61L and EGFP-Rac1Q61L mutants. Proportions of polygonal elongated cells, non-polygonal/non-elongated cells, round cells, and unclear/early spreading cells counted from images of HeLa cells seeded on poly-L-lysine coating, cultured for 24 h and 48 h, and labelled with phalloidin-TRITC staining. Parental HeLa wild type (wt) cells, negative control C23, HA-Dock10.1 expressor C33, single EGFP-Cdc42Q61L and EGFP-Rac1Q61L, and double HA-Dock10.1/EGFP-Cdc42Q61L and HA-Dock10.1/EGFP-Rac1Q61L clones were compared in the presence or absence of 2 ng/ml dox. Dox, doxycycline.

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especially the double Dock10/Cdc42 clone, potentiated the presence of non-elongated cells. These results suggest that activation of both Cdc42 and Rac1 may contribute to loss of elongation of HeLa cells induced by Dock10.

Effects of Dock10.1 and the Cdc42Q61L and Rac1Q61L mutants in cell spreading

Cell spreading was examined in clones grown on poly-L-lysine coating and labelled with phalloidin. Expression of Dock10 in C33 cells in the absence of dox induced a significant increase in the spread area of the predominant non-polygonal cells, compared to the minor fraction of non-polygonal cells in presence of dox (Fig. 6). Non-polygonal cells developed following Cdc42Q61L expression had a significant decrease in cell spreading compared to the minor fraction of non-polygonal cells, but also to the predominant fraction of polygonal cells, grown in presence of dox. The whole set of Rac1Q61L expressing cells, and both the polygonal and non-polygonal subsets, had significant increases in cell spreading in the absence of dox. HeLa cells co-expressing HA-Dock10.1 and Cdc42Q61L did not significantly alter their extension in the presence or absence of dox. However, cells co-expressing the Dock10 and Rac1 proteins had significant increases following dox withdrawal as a whole, and also for comparison between the major fraction of non-elongated cells grown in the absence of dox and the elongated cells grown in presence of dox. These results suggest that Rac1 may contribute to the increased spreading of non-elongated cells induced by Dock10.

Effects of Dock10.1 and the Cdc42Q61L and Rac1Q61L mutants on actin cytoskeleton, filopodia and membrane ruffles

Analysis of the actin cytoskeleton in the EGFP-GTPaseQ61L clones grown poly-L-lysine coating was performed by fluorescence microscopy following phalloidin-staining (Fig. 7). HeLa wt, control clone C23, and HA-Dock10.1 expressing clone C33 had been previously studied using collagen coating (Fig. 3), and actin cytoskeleton analysis (data not shown) did not differ from that using poly-L-lysine coating. Results of the latter are summarized for the predominant non-polygonal/non-elongated fractions of C33 and EGFP-GTPaseQ61L clones in the absence of dox (Fig. 7A). C33 displayed filopodia and, frequently, ruffles. The Cdc42Q61L clone frequently exhibited filopodia. The Rac1Q61L clone extensively developed ruffles. The double Dock10/GTPase clones had mixed phenotypes where the GTPase appeared to exert a dominant role: the Dock10/Cdc42 clone had increased presence of filopodia compared to C33 and the single Cdc42Q61L clone; and the Dock10/Rac1Q61L clone had increased presence of ruffles compared with the C33 clone and increased presence of filopodia compared with the single Rac1Q61L. Representative micrographs and movies illustrate these results: extensive filopodia and frequent ruffles displayed by C33 (Fig. 7B,C; supplementary material Movie 2); frequent filopodia developed by the Cdc42Q61L clone (Fig. 7D,D′; supplementary material Movie 3); abundant ruffles exhibited by the Rac1Q61L clone (Fig. 7E,E′; supplementary material Movie 4); profuse filopodia developed by the Dock10/Cdc42Q61L

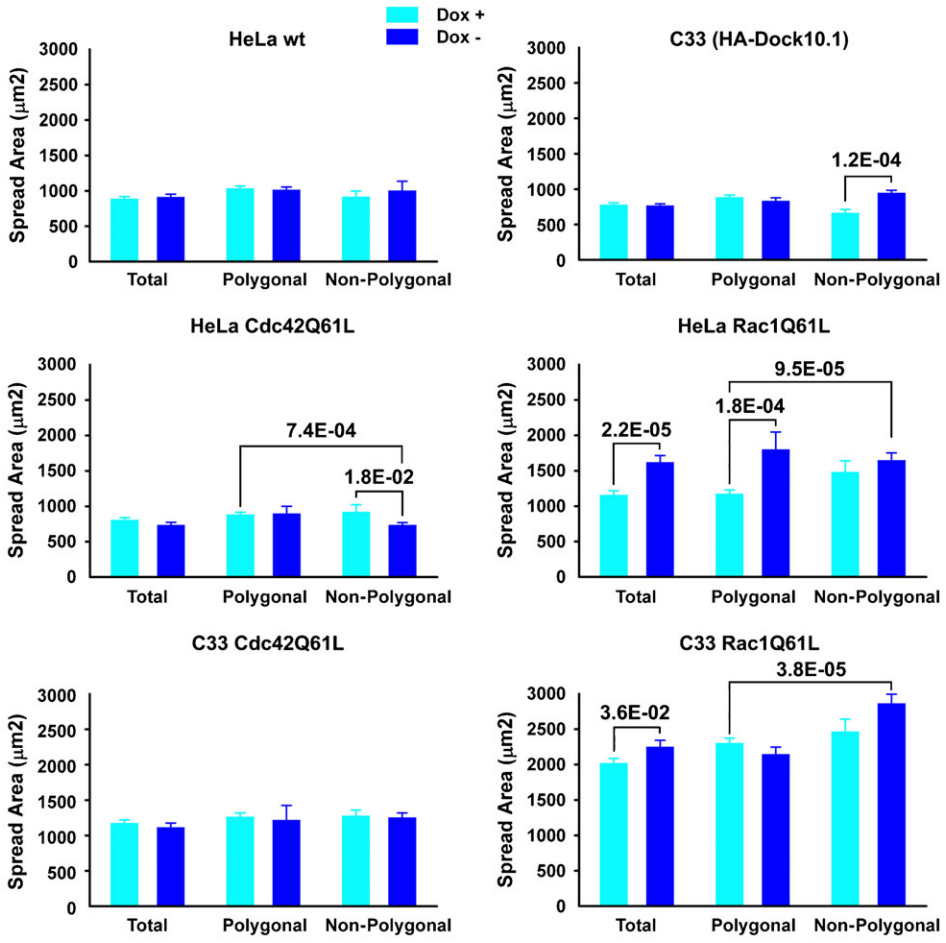

Fig. 6. Effects of expression of HA-Dock10.1 and constitutively active EGFP-Cdc42Q61L and EGFP-Rac1Q61L mutants on cell spreading. Cell area measurements (mean±s.e.m.) of total, polygonal and non-polygonal subgroups of HeLa cells and clones seeded on poly-L-lysine coated glass, cultured for 24 h and labelled with phalloidin-TRITC staining. Parental HeLa wild type (wt) cells, HA-Dock10.1 expressor C33, single EGFP-Cdc42Q61L and EGFP-Rac1Q61L, and double HA-Dock10.1/EGFP-Cdc42Q61L and HA-Dock10.1/EGFP-Rac1Q61L clones were compared in the presence or absence of 2 ng/ml dox. P values for significant differences according to Student's t test were depicted. Dox, doxycycline.

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A

%	C33 (HA-Dock10.1)	Cdc42 Q61L	Rac1 Q61L	C33 Cdc42 Q61L	C33 Rac1 Q61L
Filopodia	81.9	62.7	27.7	93.8	41.5
Ruffles	30.6	4.0	75.9	12.0	65.9

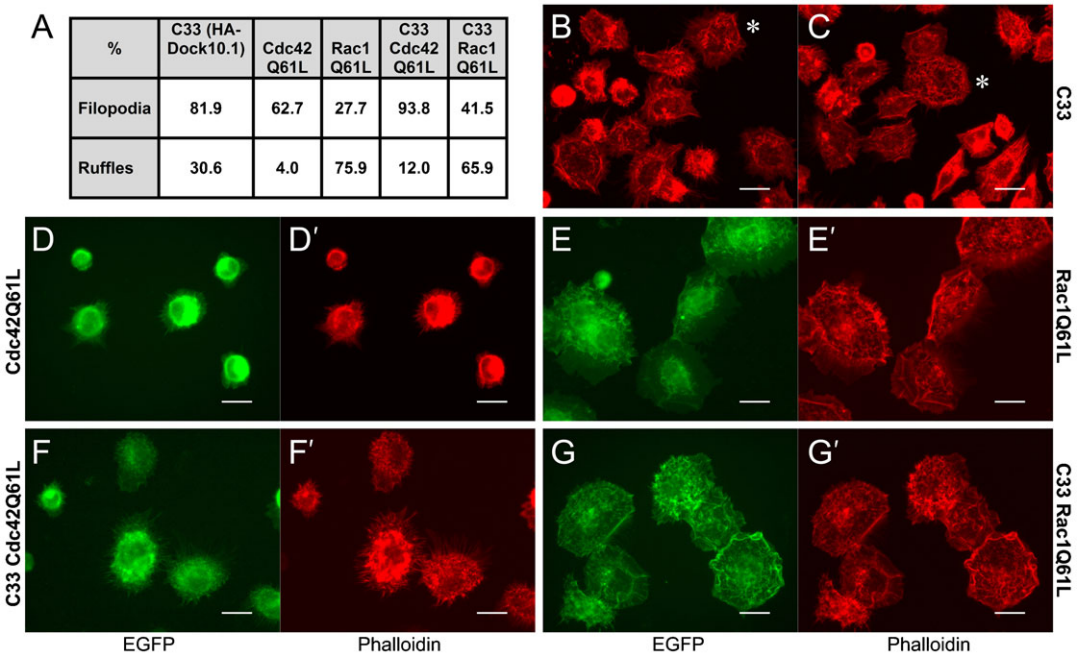

Fig. 7. Effects of HA-Dock10.1 and constitutively active EGFP-Cdc42Q61L and EGFP-Rac1Q61L mutants on actin cytoskeleton and membrane protrusions. Immunofluorescence microscopy analysis of single HA-Dock10.1, EGFP-Cdc42Q61L, EGFP-Rac1Q61L and double HA-Dock10.1/EGFP-Cdc42Q61L and HA-Dock10.1/EGFP-Rac1Q61L clones seeded onto poly-L-lysine coating after washing the cells free of dox, cultured for 24 h, and visualized with phalloidin-TRITC (red), and EGFP (green). (A) Proportions of cells rich in filopodia or ruffles within the non-polygonal cell fraction (predominant for all these clones cultured free of dox). Micrographs B and C illustrate the extensive presence of filopodia and also the frequent presence of ruffles in non-polygonal HA-Dock10.1 C33 expressing cells (ruffles prominent in cells labelled with an asterisk). Micrographs D,D′, and F,F′, illustrate the presence of filopodia in single EGFP-Cdc42Q61L and double HA-Dock10.1/EGFP-Cdc42Q61L clones, respectively. Micrographs E,E′, and G,G′, illustrate the presence of ruffles in single EGFP-Rac1Q61L and double HA-Dock10.1/EGFP-Rac1Q61L clones, respectively. Cdc42 colocalizes with filopodia and Rac1 with ruffles. Objective magnification, 40×. Scale bars, 25 μm.

(Fig. 7F,F′; supplementary material Movie 5); and last, profuse ruffles displayed by the double Dock10/Rac1Q61L clone (Fig. 7G,G′; supplementary material Movie 6). Cdc42 colocalized with filopodia and Rac1 with ruffles. These results suggest that Dock10 rearranges actin cytoskeleton by inducing filopodia through Cdc42 activation and ruffles through Rac1 activation.

DISCUSSION

Actin cytoskeleton dynamics is regulated by signaling through small Rho GTPases. In a gene silencing screen for GEFs, Dock10 was reported to function as a GEF for Cdc42 (Gadea et al., 2008). However, the study of the GTPase specificity of the Dock10 protein has been hindered by the lack of its full-length sequence. Our group has cloned two alternative first exon full-length isoforms of Dock10 (Alcaraz-García et al., 2011), thus enabling to perform such studies. In the present paper, we show that the Dock10 isoforms interact *in vitro* with nucleotide-free Cdc42 and Rac proteins, and that these interactions lead to increased activation of Cdc42 and Rac1 in HeLa cells. Thus, Dock10, which shares less homology with the other two Zizimin subfamily members, Dock9 and Dock11, and is 100 amino acids longer, also differs in its GTPase specificity, as these are Cdc42 specific. Dock10 is the third case among the Dock proteins, after Dock6 (Miyamoto et al., 2007) and Dock7 (Watabe-Uchida et al., 2006; Zhou et al., 2013), bearing dual specificity for Cdc42 and Rac proteins, whereas the rest of the members have unique specificity: Dock1 to Dock5 for Rac proteins (Kiyokawa et al., 1998; Nishihara et al., 2002; Kulkarni et al., 2011; Namekata et al., 2004; Hiramoto et al., 2006; Vives et al., 2011) and Dock8, Dock9, and Dock11, for Cdc42 (Harada et al., 2012; Meller et al., 2002; Nishikimi et al., 2005; Lin et al., 2006).

We have studied function of the Dock10.1 isoform by inducing its overexpression in adherent HeLa cells growing in planar surfaces, including tissue culture plastic, and collagen and poly-L-lysine coated glass. Our main findings are that the Dock10 protein induces a morphological change from polygonal elongated to more rounded, non-polygonal cells, which develop abundant filopodia, frequent membrane ruffles, and moderately increase their spread area in contact with the substrate. Loss of elongation was consistent with previous results by Gadea and coworkers, showing that Dock10 silencing induces the opposite effect, i.e., a change from rounded to elongated, in a melanoma cell line in three-dimensional environment (Gadea et al., 2008). In our paper, HeLa cells expressing the Cdc42 and Rac1 constitutively active mutants also induce loss of cell elongation, and this phenotype is sustained or even potentiated by co-expression of Dock10 and the GTPases, especially Cdc42. These observations are consistent with previously reported observations in Cdc42 null fibroblasts, which present with spindle shape (Czuchra et al., 2005), and in Rac1 null fibroblasts, which present with elongated shape and are defective in lamellipodia and ruffle formation (Vidali et al., 2006). Therefore, Dock10.1 may induce loss of cell elongation through both Cdc42 and Rac1.

The morphological change induced by Dock10.1 is not accompanied by a global change in the cell area in contact with the surface, suggesting that transition from elongated to non-elongated does not imply a loss of spreading capacity. In fact, our results even show that non-elongated cells expressing Dock10

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spread more than non-elongated cells under Dock10 repression by dox. Previous reports indicate that Cdc42 and Rac1 play roles in cell spreading (Price et al., 1998; Wells et al., 2004; Czuchra et al., 2005). Our results show that constitutively active Cdc42 does not induce a significant global change of cell spreading, though moderately reduce extension of non-elongated cells, and no changes are observed in the double Dock10/Cdc42 cells. In contrast, constitutively active Rac1 potentiates surface expansion, and this observation is reproduced by the double Dock10/Rac1 cells. We note that the clones may have leaky expression in the presence of dox, which may generate baseline differences. Thus, the double Dock10/Rac1 cells display the largest spread area between our clones in presence of dox, possibly due to leaky expression of Rac1 and Dock10. These results suggest that the increased spreading capacity of non-elongated cells expressing Dock10 may be mediated by Rac1.

Protrusions induced by Dock10.1, filopodia and membrane ruffles, are consistent with activation of Cdc42 and Rac1, respectively. Indeed, the constitutively active Cdc42 mutant induces filopodia, and the constitutively active Rac1 mutant, ruffles. Our data suggest that the constitutively active GTPase proteins act in a dominant fashion in the double clones, as the double Dock10/Cdc42 cells extensively exhibit filopodia while showing reduced ruffling activity, and the double Dock10/Rac1 cells extensively display ruffles while showing a substantial decrease of filopodia.

In summary, using an inducible gene expression system in a cancer adherent cell line, we present here the first cellular model for studying Dock10 function by means of its overexpression. Our data make firm the previously reported roles of Dock10 in loss of cell elongation and Cdc42 activation, and support new roles for Dock10, in Rac1 activation, induction of filopodia and membrane ruffles. Our stable Dock10.1 inducible transfectant cell lines will be valuable for investigating the functions of this regulator of small GTPases in different environments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.P. was the principal investigator, designed and performed experiments, wrote the paper, and takes primary responsibility for the paper; N.R.-L, M.-J.A.-G., and A.-M.G.-S. designed and performed experiments; S.S.-R. performed experiments; M.-R.M.-Q. performed sequencing of the plasmids. A.-M.G.-A. supervised the research. N.R.-L, and M.-J.A.-G. contributed equally to the work.

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References

Alcaraz-García, M. J., Ruiz-Lafuente, N., Sebastián-Ruiz, S., Majado, M. J., González-García, C., Bernardo, M. V., Alvarez-López, M. R. and Parrado, A. (2011). Human and mouse DOCK10 splicing isoforms with alternative first coding exon usage are differentially expressed in T and B lymphocytes. *Hum. Immunol.* **72**, 531-537.

Aspenström, P. (1999). Effectors for the Rho GTPases. *Curr. Opin. Cell Biol.* **11**, 95-102.

Aspenström, P., Fransson, A. and Saras, J. (2004). Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem. J.* **377**, 327-337.

Bernardo, M. V., Yelo, E., Gimeno, L., Campillo, J. A. and Parrado, A. (2007). Identification of apoptosis-related PLZF target genes. *Biochem. Biophys. Res. Commun.* **359**, 317-322.

Chhabra, E. S. and Higgs, H. N. (2007). The many faces of actin: matching assembly factors with cellular structures. *Nat. Cell Biol.* **9**, 1110-1121.

Czuchra, A., Wu, X., Meyer, H., van Hengel, J., Schroeder, T., Geffers, R., Rottner, K. and Brakebusch, C. (2005). Cdc42 is not essential for filopodium formation, directed migration, cell polarization, and mitosis in fibroblastoid cells. *Mol. Biol. Cell* **16**, 4473-4484.

Fluge, Ø., Bruland, O., Akslen, L. A., Lillehaug, J. R. and Varhaug, J. E. (2006). Gene expression in poorly differentiated papillary thyroid carcinomas. *Thyroid* **16**, 161-175.

Gadea, G., Sanz-Moreno, V., Self, A., Godi, A. and Marshall, C. J. (2008). DOCK10-mediated Cdc42 activation is necessary for amoeboid invasion of melanoma cells. *Curr. Biol.* **18**, 1456-1465.

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89**, 5547-5551.

Hajdo-Milasinović, A., Ellenbroek, S. I., van Es, S., van der Vaart, B. and Collard, J. G. (2007). Rac1 and Rac3 have opposing functions in cell adhesion and differentiation of neuronal cells. *J. Cell Sci.* **120**, 555-566.

Harada, Y., Tanaka, Y., Terasawa, M., Pieczyk, M., Habiro, K., Katakai, T., Hanawa-Suetsugu, K., Kukimoto-Niino, M., Nishizaki, T., Shirouzu, M. et al. (2012). DOCK8 is a Cdc42 activator critical for interstitial dendritic cell migration during immune responses. *Blood* **119**, 4451-4461.

Heasman, S. J. and Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.* **9**, 690-701.

Hiramoto, K., Negishi, M. and Katoh, H. (2006). Dock4 is regulated by RhoG and promotes Rac-dependent cell migration. *Exp. Cell Res.* **312**, 4205-4216.

Hoppe, A. D. and Swanson, J. A. (2004). Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis. *Mol. Biol. Cell* **15**, 3509-3519.

Humtsoe, J. O., Koya, E., Pham, E., Aramoto, T., Zuo, J., Ishikawa, T. and Kramer, R. H. (2012). Transcriptional profiling identifies upregulated genes following induction of epithelial-mesenchymal transition in squamous carcinoma cells. *Exp. Cell Res.* **318**, 379-390.

Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T. and Matsuda, M. (1998). Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Dev.* **12**, 3331-3336.

Kulkarni, K., Yang, J., Zhang, Z. and Barford, D. (2011). Multiple factors confer specific Cdc42 and Rac protein activation by dedicator of cytokinesis (DOCK) nucleotide exchange factors. *J. Biol. Chem.* **286**, 25341-25351.

Kurokawa, K., Itoh, R. E., Yoshizaki, H., Nakamura, Y. O. and Matsuda, M. (2004). Coactivation of Rac1 and Cdc42 at lamellipodia and membrane ruffles induced by epidermal growth factor. *Mol. Biol. Cell* **15**, 1003-1010.

Lin, Q., Yang, W., Baird, D., Feng, Q. and Cerione, R. A. (2006). Identification of a DOCK180-related guanine nucleotide exchange factor that is capable of mediating a positive feedback activation of Cdc42. *J. Biol. Chem.* **281**, 35253-35262.

Meller, N., Irani-Tehrani, M., Kiosses, W. B., Del Pozo, M. A. and Schwartz, M. A. (2002). Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins. *Nat. Cell Biol.* **4**, 639-647.

Meller, N., Irani-Tehrani, M., Ratnikov, B. I., Paschal, B. M. and Schwartz, M. A. (2004). The novel Cdc42 guanine nucleotide exchange factor, zizimin1, dimerizes via the Cdc42-binding CZH2 domain. *J. Biol. Chem.* **279**, 37470-37476.

Meller, N., Merlot, S. and Guda, C. (2005). CZH proteins: a new family of Rho-GEFs. *J. Cell Sci.* **118**, 4937-4946.

Miyamoto, Y., Yamauchi, J., Sanbe, A. and Tanoue, A. (2007). Dock6, a Dock-C subfamily guanine nucleotide exchanger, has the dual specificity for Rac1 and Cdc42 and regulates neurite outgrowth. *Exp. Cell Res.* **313**, 791-804.

Monypenny, J., Zicha, D., Higashida, C., Oceguera-Yanez, F., Narumiya, S. and Watanabe, N. (2009). Cdc42 and Rac family GTPases regulate mode and speed but not direction of primary fibroblast migration during platelet-derived growth factor-dependent chemotaxis. *Mol. Cell. Biol.* **29**, 2730-2747.

Namekata, K., Enokido, Y., Iwasawa, K. and Kimura, H. (2004). MOCA induces membrane spreading by activating Rac1. *J. Biol. Chem.* **279**, 14331-14337.

Neudauer, C. L., Joberty, G., Tatsis, N. and Macara, I. G. (1998). Distinct cellular effects and interactions of the Rho-family GTPase TC10. *Curr. Biol.* **8**, 1151-1160.

Nishihara, H., Maeda, M., Oda, A., Tsuda, M., Sawa, H., Nagashima, K. and Tanaka, S. (2002). DOCK2 associates with CrkL and regulates Rac1 in human leukemia cell lines. *Blood* **100**, 3968-3974.

Nishikimi, A., Meller, N., Uekawa, N., Isobe, K., Schwartz, M. A. and Maruyama, M. (2005). Zizimin2: a novel, DOCK180-related Cdc42 guanine nucleotide

exchange factor expressed predominantly in lymphocytes. *FEBS Lett.* **579**, 1039-1046.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Parrado, A., Noguera, M. E., Delmer, A., McKenna, S., Davies, J., Le Gall, I., Bentley, P., Whittaker, J. A., Sigaux, F., Chomienne, C. et al. (2000). Deregulated expression of leukemia translocation protein PLZF in B-cell chronic lymphocytic leukemias (B-CLL) does not affect cyclin A expression. *Hematol. J.* **1**, 15-27.

Price, L. S., Leng, J., Schwartz, M. A. and Bokoch, G. M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol. Biol. Cell* **9**, 1863-1871.

Roberts, P. J., Mitin, N., Keller, P. J., Chenette, E. J., Madigan, J. P., Currin, R. O., Cox, A. D., Wilson, O., Kirschmeier, P. and Der, C. J. (2008). Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. *J. Biol. Chem.* **283**, 25150-25163.

Rossman, K. L., Der, C. J. and Sondek, J. (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* **6**, 167-180.

Steffen, A., Ladwein, M., Dimchev, G. A., Hein, A., Schwenkmezger, L., Arens, S., Ladwein, K. I., Margit Holleboom, J., Schur, F., Victor Small, J. et al. (2013). Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. *J. Cell Sci.* **126**, 4572-4588.

Subauste, M. C., Von Herrath, M., Benard, V., Chamberlain, C. E., Chuang, T. H., Chu, K., Bokoch, G. M. and Hahn, K. M. (2000). Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. *J. Biol. Chem.* **275**, 9725-9733.

Vidali, L., Chen, F., Cicchetti, G., Ohta, Y. and Kwiatkowski, D. J. (2006). Rac1-null mouse embryonic fibroblasts are motile and respond to platelet-derived growth factor. *Mol. Biol. Cell* **17**, 2377-2390.

Vives, V., Laurin, M., Cres, G., Larrousse, P., Morichaud, Z., Noel, D., Côté, J. F. and Blangy, A. (2011). The Rac1 exchange factor Dock5 is essential for bone resorption by osteoclasts. *J. Bone Miner. Res.* **26**, 1099-1110.

Watabe-Uchida, M., John, K. A., Janas, J. A., Newey, S. E. and Van Aelst, L. (2006). The Rac activator DOCK7 regulates neuronal polarity through local phosphorylation of stathmin/Op18. *Neuron* **51**, 727-739.

Wells, C. M., Walmsley, M., Ooi, S., Tybulewicz, V. and Ridley, A. J. (2004). Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. *J. Cell Sci.* **117**, 1259-1268.

Wennerberg, K. and Der, C. J. (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). *J. Cell Sci.* **117**, 1301-1312.

Yang, L., Wang, L. and Zheng, Y. (2006). Gene targeting of Cdc42 and Cdc42GAP affirms the critical involvement of Cdc42 in filopodia induction, directed migration, and proliferation in primary mouse embryonic fibroblasts. *Mol. Biol. Cell* **17**, 4675-4685.

Yelo, E., Bernardo, M. V., Gimeno, L., Alcaraz-García, M. J., Majado, M. J. and Parrado, A. (2008). DOCK10, a novel CZH protein specifically induced by IL4 in B lymphocytes. *Mol. Immunol.* **45**, 3411-3418.

Zhou, Y., Johnson, J. L., Cerione, R. A. and Erickson, J. W. (2013). Prenylation and membrane localization of Cdc42 are essential for activation by DOCK7. *Biochemistry* **52**, 4354-4363.

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Supplementary Material

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Table S1. See supplementary webpage

Table S2. Antibodies used in this work

Protein target (clone)	Dilution (units)	Dilution (ratio)	Source	Used for	Reference	Manufacturer
Dock9	0.04 μg/ml	1:5000	Rabbit	WB	A300-530A	Bethyl Laboratories
Dock10	0.2 μg/ml	1:5000	Rabbit	WB	A301-305A	Bethyl Laboratories
Dock10.2		1:5000	Rabbit	WB	Yelo, et al., 2008	Our lab
Dock11	0.2 μg/ml	1:5000	Rabbit	WB	A301-639A	Bethyl Laboratories
Cdc42	0.25 μg/ml	1:1000	Mouse	WB	ACD03	Cytoskeleton
Rac1	0.5 μg/ml	1:1000	Mouse	WB	ARC03	Cytoskeleton
Rac (23A8)	1 μg/ml	1:1000	Mouse	WB	05-389	Upstate Technologies
Flag tag (M2)	0.2 μg/ml	1:5000	Mouse	WB	F3165	Sigma-Aldrich
HA tag (3F10)	1 μg/ml	1:100	Rat	IF	11 867 423 001	Roche Applied Science
HA-tag-HRP (3F10)	5 ng/ml	1:5000	Rat	WB	12 013 819 001	Roche Applied Science
GAPDH-HRP (FL-335)	0.2 μg/ml	1:1000	Rabbit	WB	sc-25778	Santa Cruz Laboratories
EGFP (JL-8)	1 μg/ml	1:1000	Mouse	WB	632381	Clontech
Anti-mouse-HRP	0.5 μg/ml	1:2000	Goat	WB	P0447	Dako
Anti-rabbit-HRP	0.17 μg/ml	1:2000	Swine	WB	P0399	Dako
Anti-rat-Alexa Fluor 488	2 μg/ml	1:100	Goat	IF	A11006	Invitrogen

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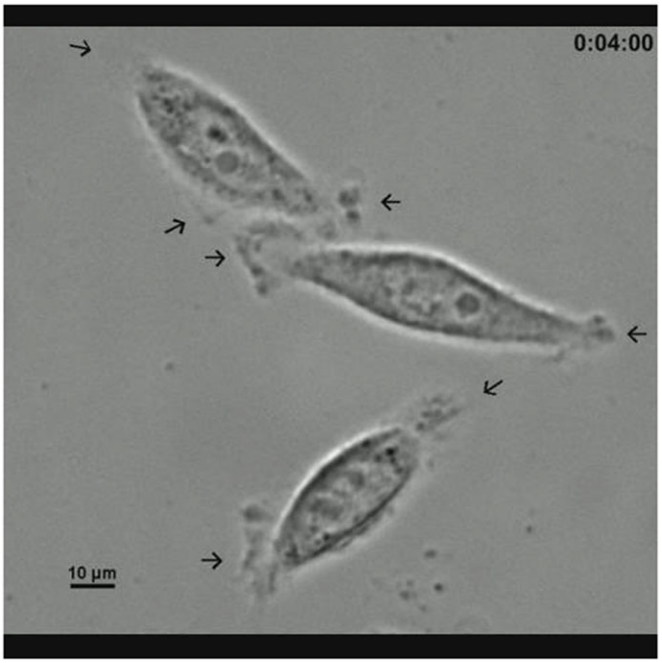
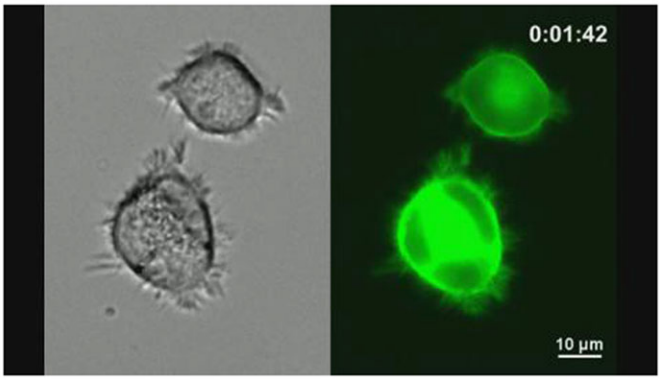
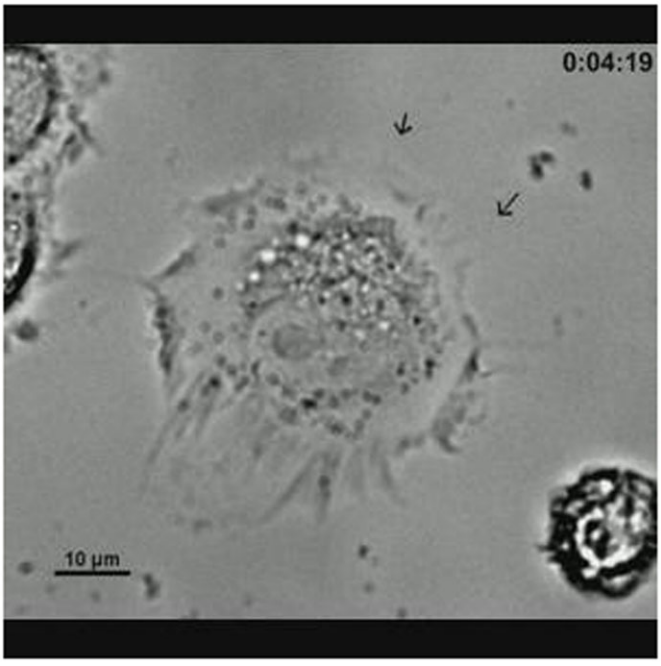


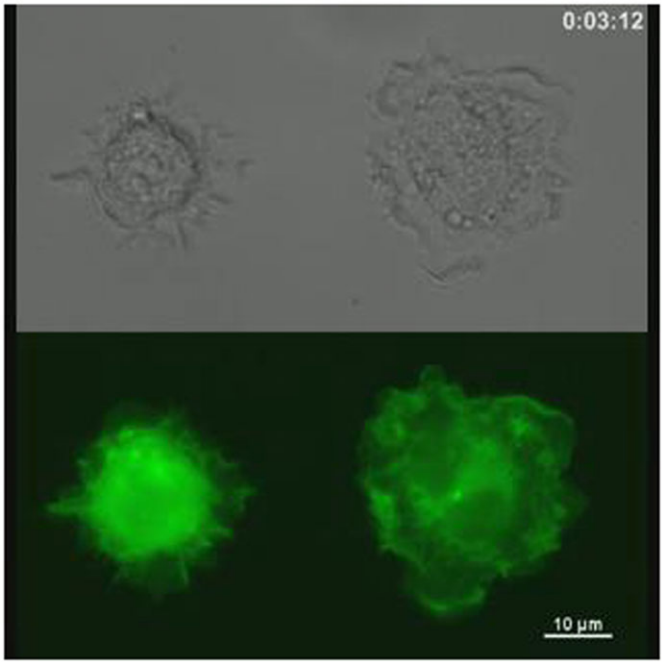
Movie 1. HeLa cells (wt) seeded on a poly-L-lysine-coated coverslip placed into a 24-well plate were cultured for 24 h, and then mounted in a heated stage chamber. Phase contrast images were registered every 20 s for 10 min in a Nikon Eclipse T*i* inverted microscope. Protrusive membrane activity at the cell vertices is depicted by arrows.



Movie 3. HeLa cell clone expressing EGFP-Cdc42Q61L processed as for previous movies. Phase contrast (left) and EGFP (right) images were registered. Non-elongated cells display filopodia.



Movie 2. HeLa cell clone C33 cells expressing HA-Dock10.1 processed as for supplementary material Movie 1. Movie shows a non-elongated flattened cell (center) displaying filopodia and ruffles. Ruffles are indicated by arrows.



Movie 4. HeLa cell clone expressing EGFP-Rac1Q61L, processed as for previous movies. Phase contrast (top) and EGFP (bottom) images were registered. Non-elongated flattened cells exhibit extensive ruffling activity.

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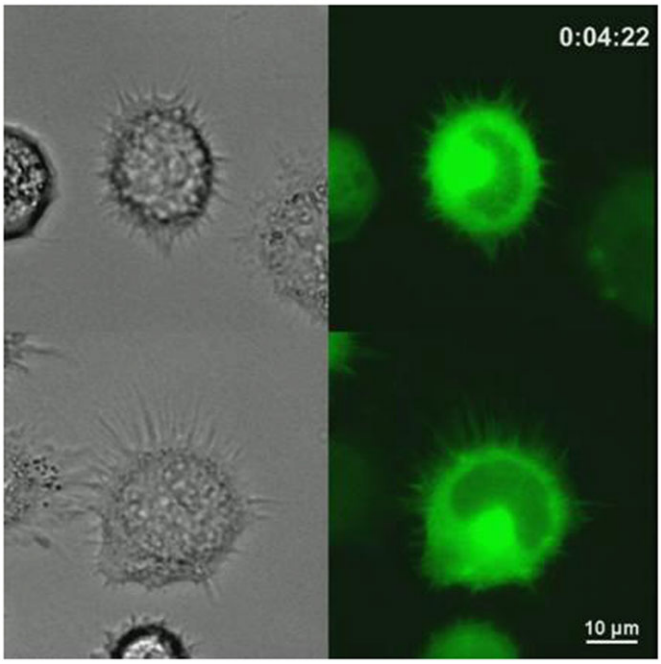
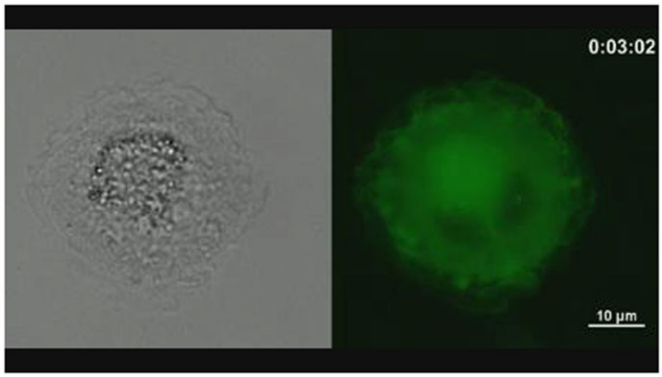


Movie 5. HeLa cell clone HeLa cell clone co-expressing HA-Dock10.1 and EGFP-Cdc42Q61L, processed as for previous movies. Phase contrast (left) and EGFP (right) images were registered. Non-elongated cells profusely display filopodia.



Movie 6. HeLa cell clone HeLa cell clone co-expressing HA-Dock10.1 and EGFP-Rac1Q61L, processed as for previous movies. Phase contrast (left) and EGFP (right) images were registered. Movie shows a non-elongated flattened cell exhibiting extensive ruffling activity.

Supplementary Table S1. Plasmids used in this work

Vector	NCBI acc. no.	Generation
Bacterial production of N-terminal GST-fusion recombinant protein		
pGEX-Cdc42	NM_044472.2	RT-PCR amplification of Cdc42 from PBMC using 5'-TTCCCGGGGCAGACAATTAAGTGTGTTG-3' and 5'-TTGCGGCCGCTTAGAATATACAGCACTTCC-3', and ligation into SmaI/NotI sites of pGEX-4T-1 (GE Healthcare)
pGEX-Rac1	NM_006908.4	RT-PCR amplification of Rac1 from PBMC using 5'-TTCCCGGGGCAGGCCATCAAGTGTGTGG-3' and 5'-TTGCGGCCGCTTACAACAGCAGGCATTTTC-3' and ligation into SmaI/NotI sites of pGEX-4T-1
pGEX-Rac2	NM_002872.4	PCR amplification of YFP-Rac2 (Addgene plasmid 11393, Hoppe&Swanson, 2004) using 5'-TTGAATTCATGCAGGCCATCAAGTGTGTGGTGG -3' and 5'-TTGCGGCCGCCTAGAGGAGGCTGCAGGCGCGCTTC-3', and ligation into EcoRI/NotI sites of pGEX-4T-1
pGEX-Rac3	NM_005052.2	PCR amplification of LZRS-MS-IRES-ZEO/pBR-Rac3 (Hajdo-Milasinović et al., 2007) using 5'-TTGAATTCATGCAGGCCATCAAGTGCGTGGTGG-3' and 5'- TTGCGGCCGCCTAGAAGACGGTGCACTTCTTCCCC-3', and ligation into EcoRI/NotI sites of pGEX-4T-1
pGEX-RhoA	NM_001664.2	RT-PCR amplification of RhoA from PBMC using 5'-GAATTCATGGCTGCCATCCGGAAGAAAC-3' and 5'-TTGCGGCCGCTTAGAATATACAGCACTTCC-3', and ligation into EcoRI/NotI sites of pGEX-4T-1
pGEX-RhoD	NM_014578.3	PCR amplification of EGFP-hRhoD (Addgene plasmid 23235, Roberts et al, 2008) using 5'-TTGAATTCATGACGGCGGCCCAGGCCGCGGGTG-3' and 5'-TTGCGGCCGCTCAGGTCACCACGCAAAAGCCCTGG-3', and ligation into EcoRI/NotI sites of pGEX-4T-1
pGEX-RhoF-SAAX	NM_019034.2	PCR amplification of pGEX-2T-RhoF (gift of Harry Mellor, University of Bristol, UK) introducing amino acid change C208S using 5'-TTGAATTCATGGATGCCCCCGGGGCCCTGGCCC-3' and 5'-TTGCGGCCGCTCAGAGCAGCAGGGAGAGCCGGCGC-3', and ligation into EcoRI/NotI sites of pGEX-4T-1
pGEX-RhoG-SAAX	NM_001665.3	RT-PCR amplification of RhoG from PBMC introducing amino acid change C188S using 5'-TTGAATTCATGCAGAGCATCAAGTGCGTGGTGG-3' and 5'-TTGCGGCCGCCTACAAGAGGATGGAGGACCGCCCA-3', and ligation into EcoRI/NotI sites of pGEX-4T-1
pGEX-RhoJ	NM_020663.3	RT-PCR amplification of RhoJ from PBMC using 5'-TTGAATTCATGAACTGCAAAGAGGGAACTGACA-3' and 5'- TTGCGGCCGCTCAGATAATTGAACAGCAGCTGTGA-3', and ligation into EcoRI/NotI sites of pGEX-4T-1
pGEX-2T-RhoQ	NM_012249.3	Neudauer et al., 1998
pGEX-PAK1	NM_002576.3	RT-PCR amplification of PAK1-PBD (amino acids 67-150) from human brain using 5'-TTCCCGGGGAAGAAAGAGAAAGAGCGGCC-3' and 5'-TTGCGGCCGCTCAAGCTGACTTATCTGTAAAGC-3', and ligation into SmaI/NotI sites of pGEX-4T-1
Eukaryotic expression, transient		
pSG5a		New MCS (AgeI-EcoRI-NotI) for pSG5 vector (Stratagene)

		by PCR amplification of pSG5 with 5'-TTGAATTCGCGGCCGCTATTAAAGCAGAACTTGTTTATTGCA-3' and 5'-TTGAATTCACCGGTTATAGTGAGTCGTATTACAATTCT-3', EcoRI digestion, and religation of vector
pSG5b		New MCS (BamHI-EcoRI-SacII) for pSG5 vector by PCR amplification of pSG5 with 5'-TTGAATTCGGATCCTATTAAAGCAGAACTTGTTTATTGCA 3' and 5'-TTGAATTCCCGCGGTATAGTGAGTCGTATTACAATTCT-3', EcoRI digestion, and religation of vector
pEF-FLAG-DOCK9	NM_015296.2	Meller et al., 2004
pSG5-DOCK10.1	NM_014689	Subcloning of DOCK10.1 from pJAG4-DOCK10.1 (this work) into AgeI/NotI sites of pSG5a
pSG5-HA-DOCK10.1	NM_014689	Subcloning of HA-DOCK10.1 from pJAG4-HA-DOCK10.1 (this work) into the AgeI/NotI sites of pSG5a
pSG5-DOCK10.2	NM_001290263.1	Subcloning of DOCK10.2 from pJAG4-DOCK10.2 (this work) into the AgeI/NotI sites of pSG5a
pSG5-HA-DOCK10.2	NM_001290263.1	PCR amplification of pJAG4-HA-DOCK10.2 (this work) using 5'-TTACCGGTAGCGCCGCCATGGAG-3' and 5'-TGTGAAGGAAGCTTCTCTGGT-3', excision of AgeI/EcoRV fragment of pSG5-DOCK10.2 (this work), and ligation of PCR fragment into AgeI/EcoRV sites
pSG5-DOCK11	NM_144658.3	Subcloning of DOCK11 from pJEF4-DOCK11 (this work) into SacII/BamHI sites of pSG5b
pSG5-HA-DOCK11	NM_144658.3	Subcloning of HA-DOCK11 from pJEF4-HA-DOCK11 (this work) into SacII/BamHI sites of pSG5b
Eukaryotic expression, stable inducible		
pUHD-15-1-Puro		Gift from Berthold Henglein (Institut Curie, Paris, France) (Bernardo et al, 2007)
pUHC-13-3		Gossen&Bujard, 1992
pJAG1		New MCS (SacII-EcoRI-AflII-Eco47III-SnaBI-SpeI-SalI-MluI-XbaI-EcoRV-AgeI-NheI-NotI-ApaI-SbfI-BamHI) for pJEF4 , gift of J.E. Floettmann & M. Rowe (University of Wales, Cardiff, UK) (Parrado et al, 2000) by insertion of synthetic oligonucleotide, ligated following excision of EcoRI/BamHI fragment
pJAG2		Exchange of Neomycin for Zeocin resistance in pJAG1 (this work) by excision of Neomycin resistance cassette with XhoI, PCR amplification of Zeocin resistance cassette of pSV40-Zeo2 (Invitrogen) using 5'-AGCTCGAGGGTGTGGAAAGT-3' and 5'-TTCTCGAGAGACATGATAAGATACATTG-3', and ligation into XhoI site
pJAG4		Modification of MCS of pJAG1 (this work) by excision of Eco47III//EcoRV fragment and religation of vector
pJAG4-DOCK10.1	NM_014689	PCR amplification of pCR2.1-hDOCK10.1 (Alcaraz-García et al., 2011) using 5'-TTACCGGTTGACCGGCGATGGCCGGTGA-3' and 5'-TAGCGGCCGCCCTCAGACTTCAGCACTA-3', and ligation into AgeI/NotI sites of pJAG4
pJAG4-HA-DOCK10.1	NM_014689	Insertion of HA tag into pJAG4-DOCK10.1 (this work) by excision of AgeI/EcoRV fragment from pJAG4-DOCK10.1, PCR amplification of pJAG4-DOCK10.1 using 5'-TTACCGGTAGCGCCGCCATGGAGTACCCATACGACGTA

		CCAGATTACGCTGCCGGTGAGCGG-3' and 5'-TGTGAAGGAAGCTTCTCTGGT-3', and ligation of PCR fragment into AgeI/EcoRV sites
pJAG4-DOCK10.2	NM_001290263.1	PCR amplification of pCR2.1-hDOCK10.2 (Alcaraz-García et al., 2011) using 5'-TTACCGGTAGCAATACGATGAGTTTTC-3' and 5'-TGTGAAGGAAGCTTCTCTGGT-3', excision of AgeI/EcoRV fragment from pJAG4-DOCK10.1, and ligation of PCR fragment into AgeI/EcoRV sites
pJEF4-DOCK10.2*	Genbank EU236710.1	Old version of pJEF4-DOCK10.2 (*; contains mutations) by RT-PCR amplification of human B cells using 5'-TTCCGCGGAGCAATACGATGAGTTTTC-3' and 5'-AACCGCGGTCAGACTTCAGCACTAGATG-3' and ligation into SacII site of pJEF4
pJEF4-HA-DOCK10.2*	Genbank EU236710.1	Insertion of HA tag in old version of pJEF4-DOCK10.2 (*; contains mutations) by PCR amplification of pJEF4-DOCK10.2* using 5'-ATCGCCTGGAGACGCCATCCACG-3' and 5'-GTTCGTCTCTTCTTCCAAAATTCACTGGGCTCCCGTTTAAAAACCTTCCCTCGAAAACTAGCGTAATCTGGTACGTCGTATGGGTACTCCATGGCGGCGCTCCGCGGAGGCTGGATCGGTC-3', excision of EspI/EspI fragment from pJEF4-DOCK10.2*, and ligation of PCR fragment into EspI site
pJAG4-HA-DOCK10.2	NM_001290263.1	Insertion of HA tag into pJAG4-DOCK10.2 (this work) by subcloning EspI/EspI fragment of pJEF4-HA-DOCK10.2* into pJAG4-DOCK10.2
pJEF4-DOCK11	NM_144658.3	Deletion of HA tag from pJEF4-HA-DOCK11 (this work) by excision of SacII/ApaI fragment, PCR amplification of pJEF4-HA-DOCK11 using TTCCGCGGGCCGCTGCCATGGCCGAAGT and CACACCACCCTTCTGAGAAC, and ligation of PCR fragment into SacII/ApaI sites
pJEF4-HA-DOCK11	NM_144658.3	PCR amplification of pKH3-DOCK11 (Lin et al, 2006) using 5'-TTCCGCGGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTGCCGAAGTGCGCAAATTCAC-3' (containing HA tag) and 5'-TTGGATCCTCACACTTCAGCGTATCTTG-3', and ligation into SacII/BamHI sites of pJEF4; aminoacid substitution R727H by excision of AccIII/Eco47III internal fragment, RT-PCR amplification of PBMC using GGAGACGGTAGAAACAGCAC and TGTGCTGGTATCTTGTGTCA, and ligation of PCR fragment into AccIII/Eco47III sites.
pJAG2-EGFP-Cdc42Q61L	NM_001791.3	PCR amplification of pcDNA3-EGFP-Cdc42-Q61L (Addgene plasmid 12986, Subauste et al., 2000) using 5'-TTCCGCGGGCCGCCACCATGGTGAGCAAGG-3' and 5'-TTGGATCCTCATAGCAGCACACACCTGC-3', and ligation into SacII/BamHI sites of pJAG2
pJAG2-EGFP-Rac1Q61L	NM_006908.4	PCR amplification of pcDNA3-EGFP-Rac1-Q61L (Addgene plasmid 12891, Subauste et al., 2000) using 5'-TTCCGCGGGCCGCCACCATGGTGAGCAAGG-3' and 5'-TTGGATCCTTACAACAGCAGGCATTTTC-3', and ligation into SacII/BamHI sites of pJAG2

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The Gene Expression Response of Chronic Lymphocytic Leukemia Cells to IL-4 Is Specific, Depends on ZAP-70 Status and Is Differentially Affected by an NFκB Inhibitor

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Abstract

Interleukin 4 (IL-4), an essential mediator of B cell development, plays a role in survival of chronic lymphocytic leukemia (CLL) cells. To obtain new insights into the function of the IL-4 pathway in CLL, we analyzed the gene expression response to IL-4 in CLL and in normal B cells (NBC) by oligonucleotide microarrays, resulting in the identification of 232 non-redundant entities in CLL and 146 in NBC (95 common, 283 altogether), of which 189 were well-defined genes in CLL and 123 in NBC (83 common, 229 altogether) ($p<0.05$, 2-fold cut-off). To the best of our knowledge, most of them were novel IL-4 targets for CLL (98%), B cells of any source (83%), or any cell type (70%). Responses were significantly higher for 54 and 11 genes in CLL and NBC compared to each other, respectively. In CLL, ZAP-70 status had an impact on IL-4 response, since different sets of IL-4 targets correlated positively or negatively with baseline expression of ZAP-70. In addition, the NFκB inhibitor 6-Amino-4-(4-phenoxyphenethylamino)quinazoline, which reversed the anti-apoptotic effect of IL-4, preferentially blocked the response of genes positively correlated with ZAP-70 (e.g. CCR2, SUSD2), but enhanced the response of genes negatively correlated with ZAP-70 (e.g. AUH, BCL6, LY75, NFIL3). Dissection of the gene expression response to IL-4 in CLL and NBC contributes to the understanding of the anti-apoptotic response. Initial evidence of a connection between ZAP-70 and NFκB supports further exploration of targeting NFκB in the context of the assessment of inhibition of the IL-4 pathway as a therapeutic strategy in CLL, especially in patients expressing bad prognostic markers.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All microarray files are available from the Gene Expression Omnibus database (accession number GSE55288).

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Introduction

Chronic lymphocytic leukemia (CLL) is a malignant disease characterized by the proliferation of CD5+CD23+ B cells. The clinical course is heterogeneous in CLL. About half of patients live for decades and never require treatment, while the other half become symptomatic or progress to late stages of the disease and require chemotherapy. Low rate of mutation of the IGHV sequence, and high levels of expression of ZAP-70, CD38, and CD49d/ITGA4, are prognostic risk markers [1]. Despite this heterogeneity, gene expression profiles (GEP) in CLL are relatively homogeneous, considering that specific CLL signatures clearly discriminate CLL cells from B cells of other related pathologic entities and from normal B cells [2–8], whereas specific signatures for CLL prognostic groups are based on more subtle differences [9–12]. CLL cells spontaneously and rapidly die *in vitro*, because they lack essential signals provided by the natural microenvironment [13]. CLL cells interact with bone marrow stromal cells, and with T cells, antigen-presenting cells and dendritic cells within the lymph node proliferation centers (or pseudofollicles). Cytokines, chemokines, integrins, and other ligands and receptors play key roles in proliferation and survival within these cellular niches [14].

Interleukin-4 (IL-4) is a cytokine secreted by activated T cells, NK-T cells, basophils, eosinophils and mast cells. Paracrine

stimulation through the IL-4 membrane receptor (IL-4R) induces signaling cascades leading to maturation of B-cell precursors into immunoglobulin-secreting cells and antigen presenting cells, proliferation of activated B cells, and induction of isotype switching toward IgE [15]. The activated IL-4R phosphorylates JAK1 and JAK3. JAK1 phosphorylates STAT6 which homodimerizes and enter the nucleus to regulate gene expression. JAK1 and JAK3 lead to anti-apoptotic signaling through PI3K/AKT and the mitochondrial pathway, and through the Ras/MAPK pathway and NFκB activation [16]. NFκB activation is anti-apoptotic in CLL [17,18]. In B cells, IL-4 induces preferentially the non-canonical NFκB pathway [19]. IL-4 induces efficient STAT6 phosphorylation and activation in CLL [20]. However, binding of NFκB to the promoter of IGHE, CD86 and MHCII is necessary for STAT6 binding and transcription [19,21,22].

IL-4 efficiently protects CLL cells from spontaneous apoptosis or killing with agents such as fludarabine and chlorambucil [13,24,25]. CLL cells have been reported to be more prone than normal B cells (NBC) to spontaneous apoptosis [13], and those expressing good prognostic markers more than those expressing bad prognostic markers [23]. IL-4 acts in a paracrine rather than autocrine manner in CLL [26]. GEPs in follicular lymphoma suggest that a connection dependent on IL-4 between T cells and the malignant B cells sustains tumorigenesis [27]. Similarly, IL-4 could play a role in CLL pathogenesis and progression. Several studies have focused on identifying the IL-4 targets in mouse B splenocytes [28], some lymphoma subtypes [29], and other non B cell types (see additional references in Table S5). However, the gene expression response to IL-4 in CLL is poorly known.

We report here the first study aimed at identifying the IL-4 targets in CLL. We found sets of genes differentially regulated by IL-4 in CLL and NBC, and within CLL, depending on ZAP-70 expression, suggesting that the gene expression response to IL-4 may be relevant in CLL pathogenesis and prognosis. Finally, we found evidence for a dual mechanism which links the gene expression response to IL-4, NFκB activity, and ZAP-70 expression, based on the observation that a proportion of the IL-4 targets have a higher response in ZAP-70 positive patients which can be blocked by an NFκB inhibitor, and another group of IL-4 targets have a higher response in ZAP-70 negative patients which can be further induced by the NFκB inhibitor.

Methods

Sample collection

Peripheral blood samples from 38 chronic lymphocytic leukemia (CLL) patients and 13 controls with normal lymphopoiesis were obtained. The study was approved by the Review Board of Hospital Clínico Universitario Virgen de la Arrixaca, and the participants provided their written informed consent. All the patients had leukocytosis and did not receive treatment during the prior 3 months to sample collection. From the 38 CLL patients, 23 were studied by microarray and 15 were included later to increase the statistical significance of validations (Table S1).

Cell isolation

Samples were processed to isolate the B cells by negative selection procedures which were based on cocktails containing CD2, CD16, CD36 and CD235a antibodies for depletion of T

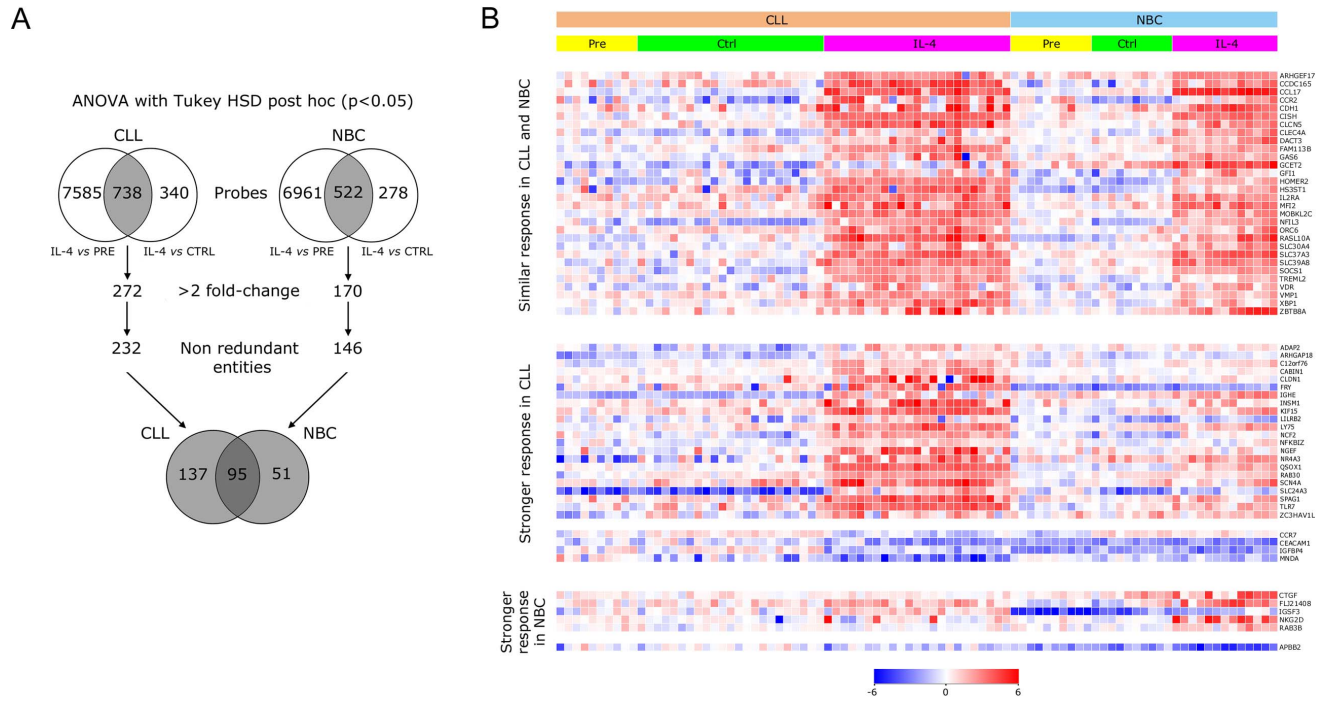

Figure 1. Identification of the IL-4 targets in CLL and in NBC. (A) Scheme of the strategy used to identify the IL-4 targets in patients and in controls. (B) Heat maps for expression of IL-4 targets which had: *top panel*, similar changes in CLL and NBC and above 3-fold change for both; *center panel*, significantly higher changes in CLL and above 3-fold change in CLL; *bottom panel*, significantly higher changes in NBC and above 3-fold change in NBC. IL-4 targets are ordered alphabetically. In the event that several probes represent the same gene, only one is shown. Relative expression levels are depicted according to the shown $\log_2$ color scale.
doi:10.1371/journal.pone.0109533.g001

Table 1. Top 50 IL4 targets in CLL and NBC.

CLL		NBC	
Gene Symbol	**Fold Change**[†]	**Gene Symbol**	**Fold Change**[†]
SLC24A3	61.79	CCL17	24.37
CCR2	34.08	CCR2	17.79
NFIL3	30.62	**NKG2D**	16.18
CCL17	19.82	NFIL3	15.33
HOMER2	18.72	CLEC4A	10.87
QSOX1	16.83	HOMER2	10.45
SOCS1	14.21	CCDC165	8.26
NCF2	13.85	CISH	7.65
RASL10A	13.56	HS3ST1	7.62
CLCN5	12.98	RASL10A	7.55
SLC39A8	12.29	SLC37A3	6.94
SLC37A3	12.00	QSOX1	6.92
NGEF	11.18	VDR	6.59
CISH	10.98	MFI2	6.51
NR4A3	10.08	SLC30A4	6.41
XBP1	9.47	PEG10	6.41
SPAG1	8.97	ENPP1	6.09
KIF15	7.57	**TMEM71**	6.06
BCL6	7.10	SLC39A8	5.91
IGHE	7.09	CLCN5	5.77
CCDC165	6.83	ZBTB8A	5.31
CLEC4A	6.65	**CTGF**	5.19
SUSD2	6.33	MOBKL2C	4.98
TLR7	6.23	NETO1	4.89
SCN4A	6.23	CDH1	4.83
MOBKL2C	6.03	GCET2	4.82
INSM1	5.90	MACROD2	4.76
ADAP2	5.89	**RAB3B**	4.60
RNF19A	5.82	FAM126A	4.33
CARD9	5.63	IL2RA	4.29
CDH1	5.62	GNG8	4.29
TREML2	5.56	IL4I1	4.25
TBC1D8	5.50	ARHGEF17	4.09
FCRL2	5.46	GAS6	4.00
CLDN1	5.45	ZNF443	3.96
LY75	5.40	**IGSF3**	3.94
SLC30A4	5.36	APOL6	3.93
AUH	5.11	PALLD	3.83
MFI2	5.08	SPINT2	3.81
GFI1	5.08	**FLJ21408**	3.79
SLC47A1	4.90	TREML2	3.76
VMP1	4.58	SOCS1	3.74
FAM126A	4.55	GFI1	3.61
CHSY1	4.53	PHF20L1	3.57
HS3ST1	4.46	KMO	3.43
GCET2	4.38	C16orf87	3.36
ZC3HAV1L	4.31	IGHE	3.31
RNF125	4.16	IL4R	3.30

Table 1. Cont.

CLL		NBC	
Gene Symbol	**Fold Change†**	**Gene Symbol**	**Fold Change†**
MNDA	−4.39	**APBB2**	−3.51
CCR7	−4.48	SLC2A5	−4.07

(†) Mean fold change for comparison IL-4 vs Ctrl.
Genes in **bold** characters: significantly higher in CLL or NBC for both comparisons (IL-4 vs Pre and IL-4 vs Ctrl).
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cells, NK cells, monocytes, macrophages, and erythrocytes. The RosetteSep Human B Cell Enrichment Cocktail kit (StemCell Technologies, Vancouver, Canada) was suitable for CLL samples, since these are rich in malignant B cells. Small volumes of peripheral blood (10 mL) were collected from patients, and B cell isolation was directly performed during Ficoll centrifugation, following the manufacturer's instructions. The Dynabeads Untouched Human B cells kit (Invitrogen, Carlsbad, CA) was a suitable choice for normal B cells (NBC) due to the low content of B cells in the peripheral blood of normal subjects (usually less than 10% of the lymphocytes). Larger volumes of peripheral blood were collected (500 mL), PBMC were isolated by centrifugation over Ficoll 1.077 g/mL, and NBC isolated using the kit, following the manufacturer's instructions. Enrichment was determined by labelling with CD19-FITC, CD3-PE, and CD5-PE-Cy7 ((BD Biosciences), followed by acquisition in a FACScalibur flow cytometer (BD Biosciences), and analysis using the CellQuest software. Purity of CD19+ cells was 93.5±1.41% (mean ±s.e.m.) in NBC and 97.54±0.34% in CLL, including 0.56±0.17% of CD19+CD5- potential normal B cells within the CLL fractions (range 0–1.98%). The percentage of ZAP-70 positive cells within the CD19+CD5+ fraction of CLL was determined from aliquots of peripheral blood subjected to red cell lysis, permeabilization with the Cytofix/Cytoperm kit, and labelling with CD19-FITC, ZAP70-PE, CD5-PE-Cy7, and CD3-APC (BD Biosciences).

Cell culture and determination of apoptosis

Following purification, three fractions of the purified CLL and NBC were processed: a) at time zero ("Pre"); b) after being cultured for 18 hours in RPMI-1640 medium supplemented with 10% fetal calf serum (Cambrex, East Rutherford, NJ) ("Ctrl"); and c) as b, but with adding 10 ng/mL of human recombinant IL-4 (BD Biosciences, San Diego, CA) ("IL-4"). In the absence of sufficient material, the Ctrl culture was not carried out in 3 NBC (NBC06, NBC07, and NBC08). In selected patients, additional fractions were treated with IL-4 plus InSolution NF-kB activation inhibitor [6-Amino-4-(4-phenoxyphenylethylamino)quinazoline] (Merck, Nottingham, UK) at 1 μM and 10 μM. Apoptosis of the cultured cells was determined by dual labelling with annexin V and propidium iodide (BD Biosciences), and flow cytometry analysis.

RNA isolation

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). RNA samples were quantitated in a NanoDrop 2000 (Thermo Fisher Scientific, Whaltham, MA). RNA quality was examined in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only samples with R.I.N. (RNA Integrity Number) >7.0 were further studied.

Microarray analysis

From each RNA sample, 250 ng were labeled with cyanine 5-CTP (Cy5) using Agilent Two Color Quick Amp Labeling and RNA Spike-In kits, according to the manufacturer's protocol. A pooled sample composed of equimolar amounts of RNA from Pre samples of 4 CLL and 4 NBC (reference), was labelled with cyanine 3-CTP (Cy3). The labeled cRNAs were mixed together and hybridized onto Agilent Whole Human Genome Microarrays (4×44 k) targeting 19,596 Entrez Gene RNAs, using the Agilent Gene Expression Hybridization kit. After hybridization, the microarray slides were washed and scanned in an Agilent G2565CA DNA Microarray Scanner. Images were analyzed with the Agilent Feature Extraction software to automatically generate the datasets. Log_{10} ratios (test vs reference) were computed after normalization correction performed by linear and Lowess methods. The datasets were statistically analyzed and visualized using the GeneSpring GX software (Agilent), using the one-way ANOVA test ($p<0.05$) between samples Pre, Ctrl, and IL-4, with post hoc Tukey HSD analysis (Figure 1A). The computation of p-values was performed using the Benjamini-Hochberg FDR correction. CLL and NBC were analyzed independently for each cell type. Those entities that passed the analysis with increases or decreases above 2-fold for comparisons IL-4 vs Pre, and IL-4 vs Ctrl concurrently and with the same direction of change were considered IL-4 targets. Similarly, entities that passed the ANOVA test with post hoc analysis with increases or decreases above 2-fold for comparisons Pre vs Ctrl, and IL-4 vs Ctrl concurrently and with the same direction of change, excluding some genes that were among the IL-4 targets, represented genes whose expression was altered by cell culture but remained stable under culture with IL-4. Finally, entities that passed the ANOVA test with post hoc analysis with increases or decreases above 2-fold for comparisons Pre vs Ctrl, and Pre vs IL-4 concurrently and with the same direction of change, excluding some genes that were in the previous two lists, represented genes whose expression was altered by cell culture and not stabilized by IL-4. Our initial analysis performed with the first 10 patients and controls showed an elevated number of entities with significant changes induced by cell culture and not stabilized by IL-4, indicating that our approach of subtracting sample Ctrl was essential to identify changes specifically induced by IL-4. However, most changes identified for comparisons IL-4 vs Ctrl were also significant for comparison IL-4 vs Pre. For this reason, comparison IL-4 vs Pre was judged as dispensable from that time. Thus, our final microarray analysis was based on 23 CLL patients (10 Pre, 23 Ctrl, and 23 IL-4), and 13 NBC samples (10 Pre, 10 Ctrl, and 13 IL-4). Cells from patient CLL01 treated with IL-4 plus NFκB inhibitor was also studied by microarray. Datasets were deposited at the Gene Expression Omnibus database under accession number GSE55288. After defining the IL-4 targets, to determine whether they had responses of different magnitude

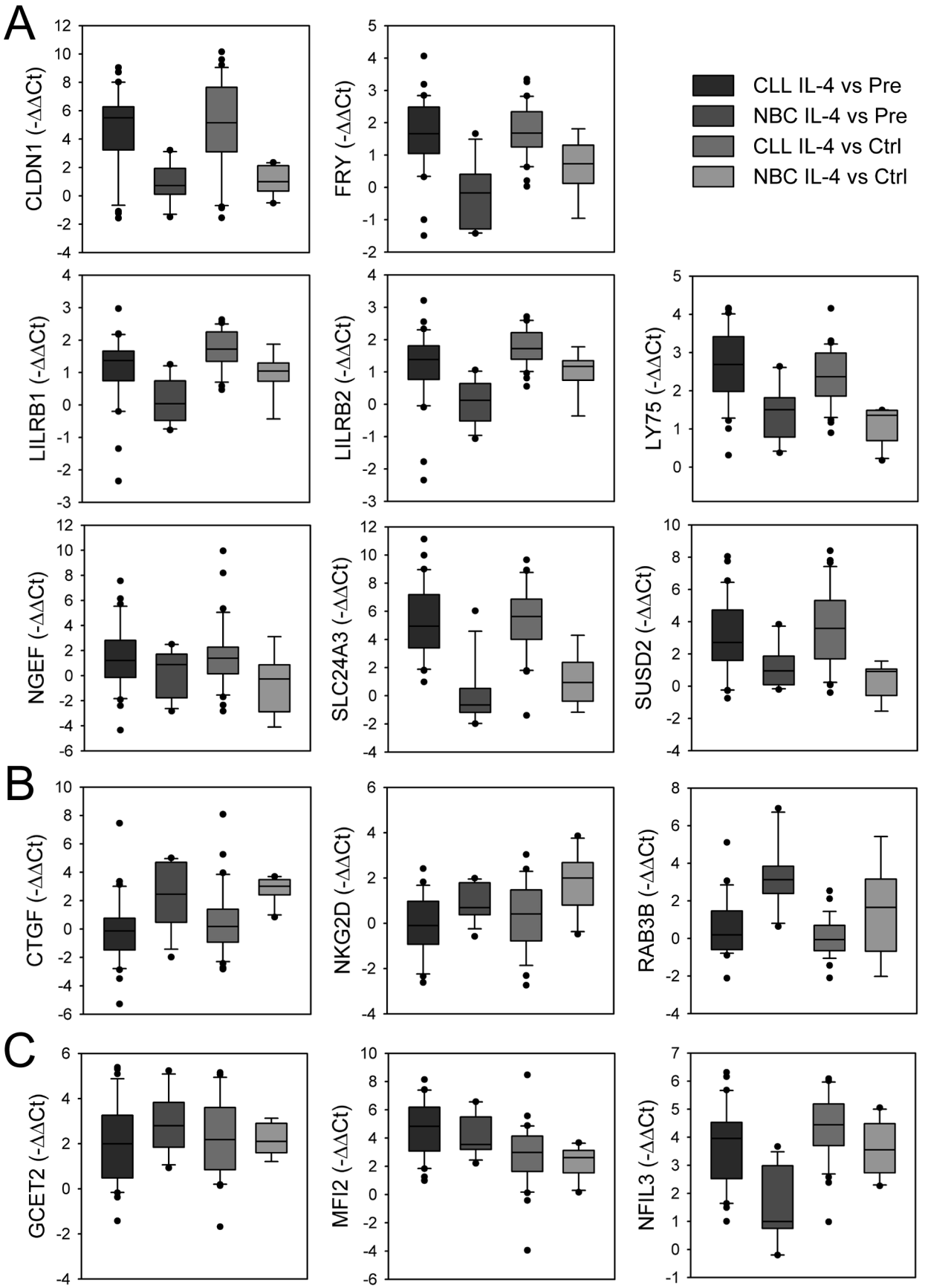
A
B
C
CLDN1 (-ΔΔCt)
FRY (-ΔΔCt)
LILRB1 (-ΔΔCt)
LILRB2 (-ΔΔCt)
LY75 (-ΔΔCt)
NGEF (-ΔΔCt)
SLC24A3 (-ΔΔCt)
SUSD2 (-ΔΔCt)
CTGF (-ΔΔCt)
NKG2D (-ΔΔCt)
RAB3B (-ΔΔCt)
GCET2 (-ΔΔCt)
MFI2 (-ΔΔCt)
NFIL3 (-ΔΔCt)
CLL IL-4 vs Pre
NBC IL-4 vs Pre
CLL IL-4 vs Ctrl
NBC IL-4 vs Ctrl

Figure 2. Validation of IL-4 targets in CLL and NBC by qPCR. Box whiskers representations of qPCR validations of 14 IL-4 targets representative of (A) restricted to CLL; (B) restricted to NBC; and (C) common to CLL and NBC. QPCR data are expressed as $-\Delta\Delta$Ct. IL-4 targets are ordered alphabetically.
doi:10.1371/journal.pone.0109533.g002

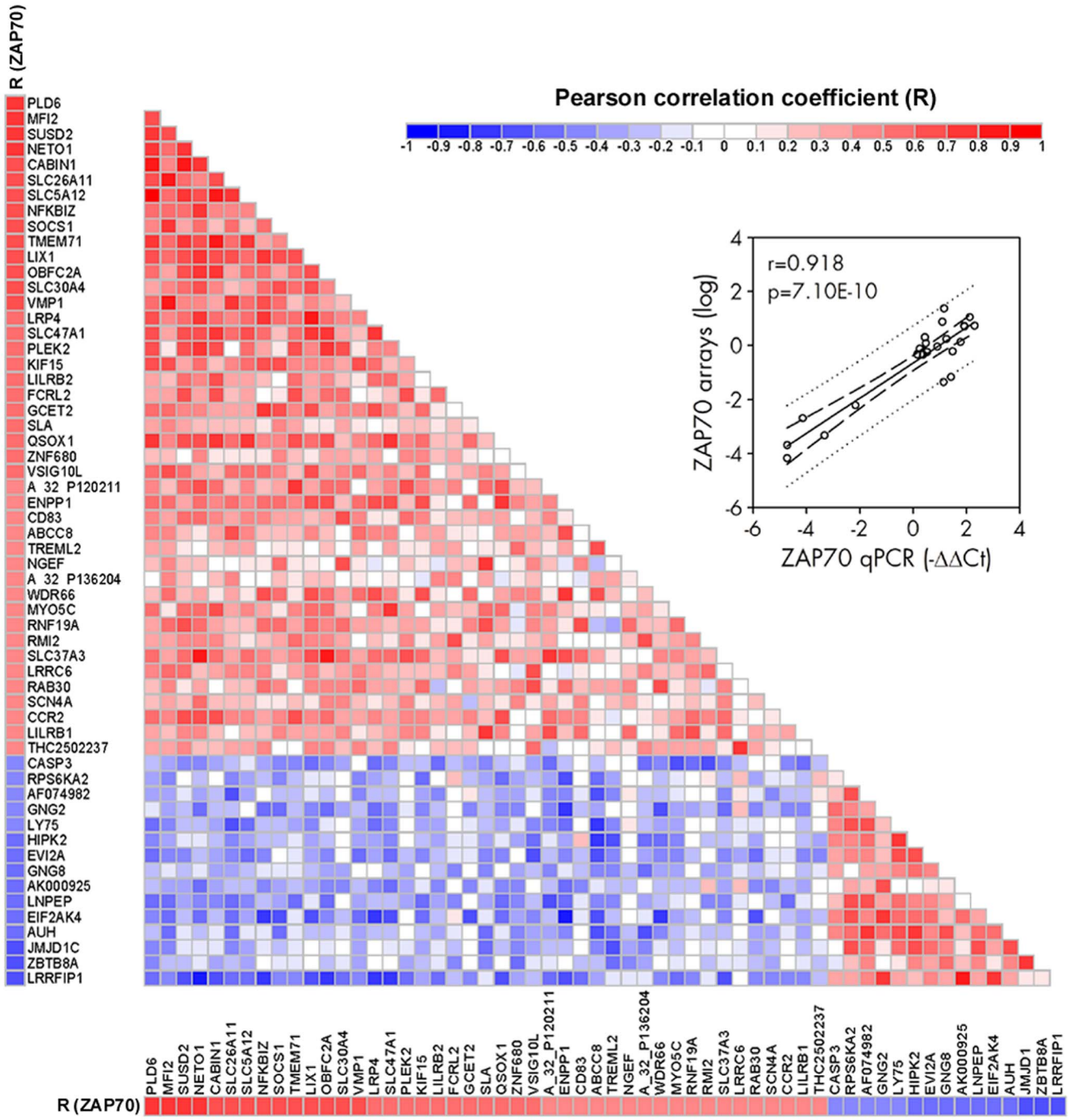

Figure 3. Pearson correlation analysis of the IL-4 upregulated targets compared between themselves and with ZAP-70. Triangular heat map representing the pairwise correlation coefficients (R) of the IL-4 upregulated targets between themselves. The IL-4 targets are ordered according to their correlation coefficients with ZAP-70, which are represented at the left and bottom sides. Cut-off values for positive or negative correlations with ZAP-70 were set at 0.4 and -0.4, respectively. In the event that several probes represent the same gene, only one is shown. Correlation coefficients are depicted according to the shown color scale. Inset shows Pearson correlation analysis between ZAP-70 levels by microarray (expressed as $\log_2$ ratios) and by qPCR (expressed as $-\Delta\Delta$Ct ratios).
doi:10.1371/journal.pone.0109533.g003

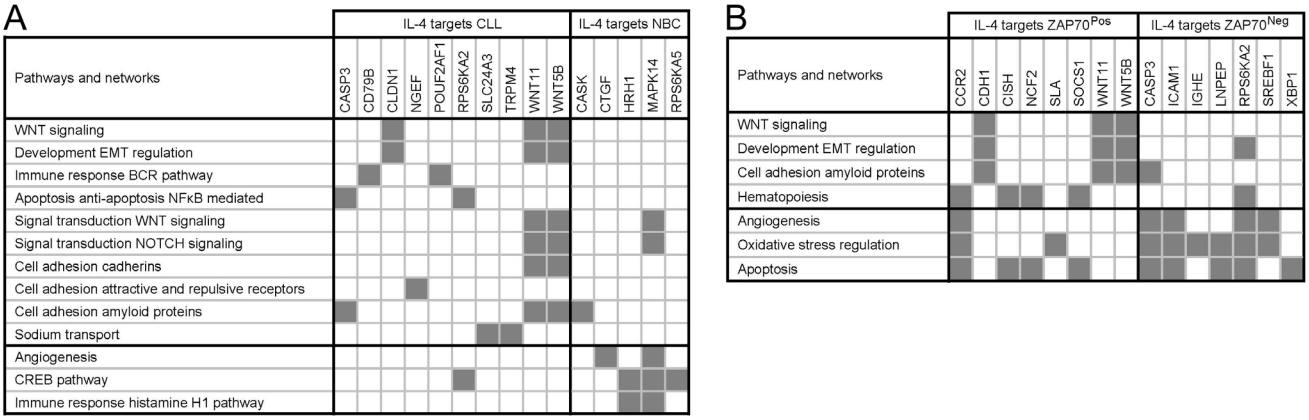

Figure 4. Pathways and networks differently affected according to MetaCore analysis of IL-4 targets. (A) Pathways and networks differently affected in CLL and NBC. (B) Pathways and networks differently affected in ZAP-70 positive and negative patients. Relevant genes for these functions within each group are specified.
doi:10.1371/journal.pone.0109533.g004

between CLL and NBC, the extent of changes in each individual (expressed as $\log_2$ ratios for IL-4 vs Ctrl) was compared by the Student t test using the SigmaStat statistical analysis package (Systat Software Inc, San Jose, CA). In addition, the behavior of the different IL-4 targets was compared between patients by Pearson correlation analysis using SigmaStat, and by hierarchical clustering analysis using the Ward's linkage method on euclidean distances. Predictions for molecular interactions, activation state of trascription factors, biological functions, canonical pathways, and process networks, interpreted from the sets of genes differentially expressed, were performed using the MetaCore software (Thomsom Reuters Systems Biology, New York City, NY).

Quantitative PCR (qPCR)

RNA samples were subjected to retrotranscription with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), following the manufacturer's instructions. QPCR was performed with the SYBR Premix Ex Taq (Takara Bio, Mountain View, CA) in an ABI Prism 7000 Sequence Detection System. QuantiTect primer assays (Qiagen) specific for the following genes were used: AUH, BCL6, CCR2, CDH1, CLDN1, CTGF, FRY, GAPDH, GCET2, INSM1, LILRB1, LILRB2, LY75, MFI2, NFIL3, NGEF, NKG2D, RAB3B, SLC24A3, SUSD2, and ZAP-70. ZAP-70 mRNA quantification via qPCR is a strong surrogate marker of IGHV mutational status and a powerful prognostic factor [30]. This method can be applied to purified B cells, and does not require an internal control (T cells) to evaluate the positivity limit. Both qPCR and microarray measures of ZAP-70 expression were used for analysis of correlations with GEPs. GAPDH was used as reference for all the qPCR assays. Measures were performed in duplicate. Analysis with the 7000 System SDS software provided the cycle threshold (Ct) values. The average Ct values for GAPDH were subtracted from the average Ct values for IL-4 target genes, resulting in the ΔCt values. Next, the ΔCt value for the reference sample was subtracted from all the others including itself, resulting in the $\Delta\Delta$Ct values. Finally, the relative expression values, expressed as fold change compared to the reference, were generated using the formula $2^{-\Delta\Delta Ct}$. For validation of microarray data, the fold changes obtained by microarray, expressed as $\log_2$, and the $-\Delta\Delta$Ct values obtained by qPCR were compared by the Student t test, and by Pearson correlation analysis, using SigmaStat.

Results

Basal GEPs of CLL and NBC

To obtain an indication that our methodological approach was reliable, the baseline GEPs of CLL and NBC were compared by the Student t test, and the genes differentially expressed were contrasted with the literature [2–12]. Many of the genes significantly overexpressed (e.g. ABCA6, FMOD, IGFBP4, IGSF3, LEF1, RASGRF1, RHOC, ROR1, WNT3), or underexpressed (e.g. EBF1, HIF1A, IQSEC1, KLF3, MS4A1, SIPA1, TRIB2, TUBB1, VAV3, ZBTB16) in CLL, were concordant with previous studies, thereby providing proof of the validity of our microarray study. The complete list (cut-off 2-fold, $p<0.05$ (Table S2)), and a heat map representation of the most significant (cut-off 3-fold, $p<0.001$ (Figure S1)), are provided. Of note, no significant difference for IL4R expression was found between CLL and NBC.

Identification of IL-4 targets in CLL and NBC

Microarray analysis identified 232 non-redundant entities in CLL (188 upregulated, 44 downregulated; Table S3), and 146 non-redundant entities in NBC (133 up, 13 down; Table S4) as IL-4 targets (cut-off 2-fold, $p<0.05$), being 95 common to both groups (90 up, 5 down), 137 restricted to CLL (98 up, 39 down), and 51 restricted to NBC (43 up, 8 down). Because an incubation period of 18 h allows regulation of direct and indirect IL-4 targets, we assume that the genes with the highest levels of change would probably be direct targets. The 50 IL-4 targets with the highest levels of change in CLL and NBC are shown in Table 1. From the 283 non-redundant entities identified as IL-4 targets in CLL and NBC altogether, 229 were well-defined genes (189 in CLL, 129 in NBC, 89 common), and the remaining corresponded to sequences not fully defined. To the best of our knowledge, 186 out of the 189 genes (98.5%) were novel IL-4 targets for CLL, 191 out of the 229 genes (83%) were novel IL-4 targets for B cells of any source, and 160 out of the 229 genes (70%), were novel IL-4 targets for any cell type (Table S5). Therefore, the vast majority of the genes identified in our study were novel IL-4 targets. The search for genes differentially regulated between CLL and NBC was refined by comparing the intensity of their changes by the Student t test ($p<0.05$). As a result, 54 genes (38 up, 16 down) had higher responses in CLL, and 11 genes (9 up, 2 down) had higher responses in NBC

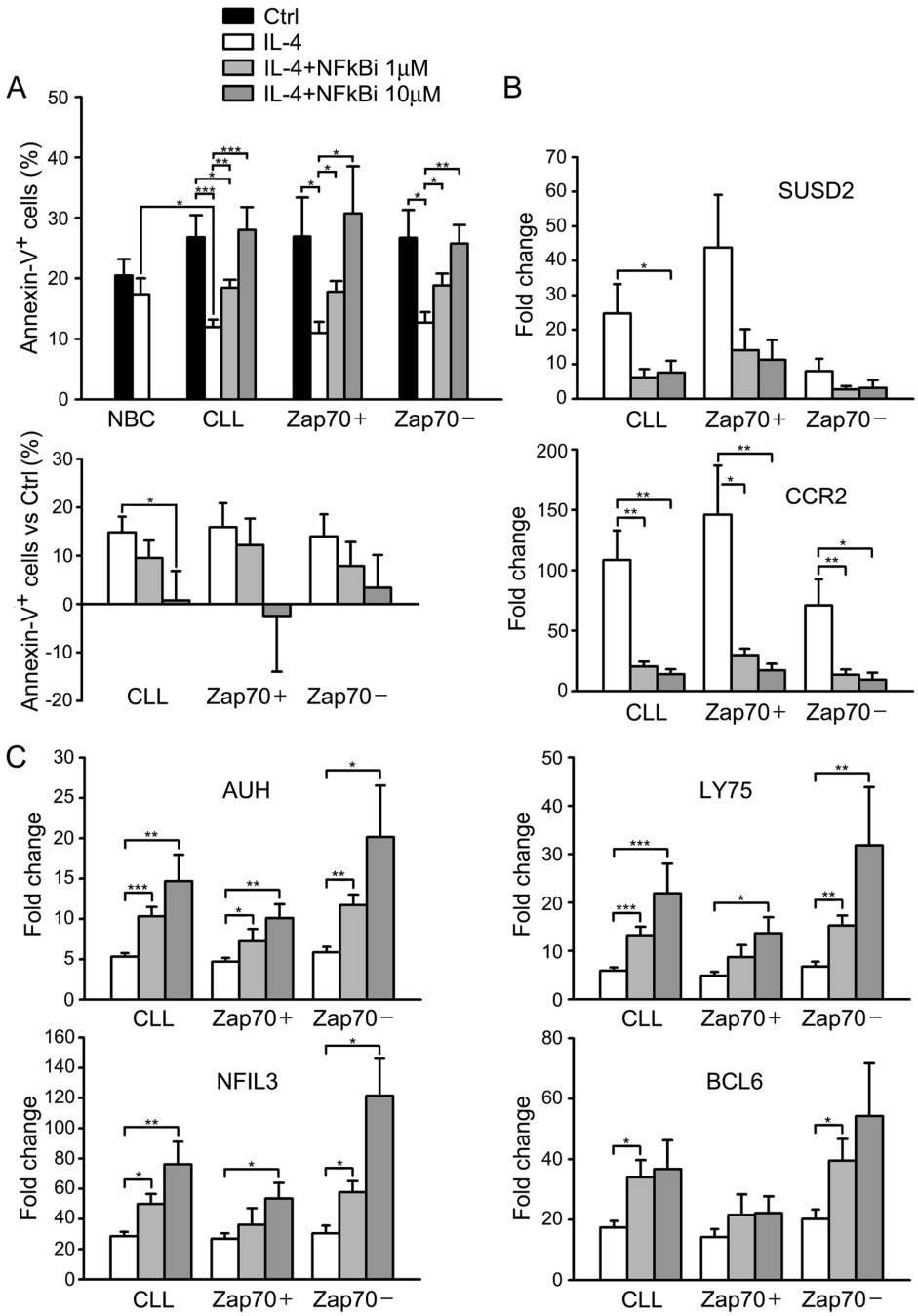

Figure 5. Effect of an NFκB inhibitor on apoptosis and gene expression response to IL-4 in CLL. (A) Apoptosis of NBC and CLL cells cultured for 18 h in the absence or presence of IL-4, and CLL cells cultured with IL-4 plus an NFκB activation inhibitor (NFκBi) at 1 μM and 10 μM. CLL are represented together and also separated in ZAP-70 positive and negative. Apoptosis was measured as the percentage of cells labelled with Annexin V (*top panel*). The *bottom panel* represents results in CLL patients after subtracting the percentage of apoptotic cells of the Ctrl samples to the IL-4, IL-4 plus 1 μM NFκBi, and IL-4 plus 10 μM NFκBi samples. T tests were used to compare the levels of apoptosis between cell types and conditions. When differences were significant the p-values are indicated as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (B) Expression of the ZAP-70^{Pos} IL-4 targets SUSD2 and CCR2, and (C) of the ZAP-70^{Neg} IL-4 targets AUH, LY75, NFIL3, and BCL6), measured by qPCR, in 7 ZAP-70 positive and 8 ZAP-70 negative patients. The ratios for expression of IL-4 targets following treatment with IL-4 alone, IL-4 plus NFκBi at 1 μM, and IL-4 plus NFκBi at 10 μM, compared to Ctrl, are represented. P-values are depicted as in A.
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(Table S3, Table S4, and Figure 1B centre and bottom showing a selection of those that changed above 3-fold). These findings suggest that IL-4 probably induces divergent pathways in CLL and NBC.

Validation of microarray analysis

To validate microarray experiments, representative IL-4 targets, restricted to CLL or NBC, or unrestricted, were assayed by qPCR (Figure 2). In most cases, comparison using the Student's t test validated microarray data. For most genes, both techniques

Table 2. IL-4 targets correlated with protection by IL-4 in CLL.

Gene Symbol	Fold change†	R-coefficient	p-value
HOMER2	18.72	0.579	3.80E-02
BCL6	7.10	0.598	3.08E-02
CLEC4A	6.65	0.657	1.47E-02
FCRL2	5.46	0.813	7.29E-04
CABIN1	4.09	0.600	3.03E-02
RMI2	3.90	0.660	1.41E-02
LILRB2	3.86	0.665	1.32E-02
FRY	3.62	0.568	4.28E-02
GIT2	2.70	0.633	2.01E-02
TMEM71	2.66	0.735	4.22E-03
BDH2	2.65	0.668	1.25E-02
PLEK2	2.59	0.579	3.80E-02
ZNF107	2.14	0.721	5.42E-03
GADD45B	-2.89	-0.564	4.48E-02

(†) Mean fold change for comparison IL-4 vs Ctrl.

doi:10.1371/journal.pone.0109533.t002

correlated significantly ($p<0.05$, Figure S2). At the protein level, other authors and ourselves have provided, in previous reports, validation for several IL-4 targets detected in this study, such as CYSLTR1 [31], IGHE [32] and NFIL3 [33] in B cells, or for DOCK10 in CLL and NBC cells [34].

Correlations between IL-4 targets, and with basal expression of ZAP-70

To determine whether the gene expression response to IL-4 was homogeneous between patients, a correlation analysis between changes of every pair of genes, and between changes of each gene and the basal levels of expression of ZAP-70, CD38, and ITGA4 (whose levels did not significantly change under culture or treatment with IL-4) were performed. The numbers of non-redundant entities correlated positively or negatively with ZAP-70 were 42 and 20, respectively, setting the cut-off values of R-coefficient at ± 0.4 (Figure 3; the R-coefficients for all the IL-4 targets are shown in Table S10). Expression of ZAP-70 determined by microarray and qPCR were correlated (Figure 3 inset). This analysis indicates that specific sets of genes were preferentially induced in the ZAP-70 positive or negative patients. According to this criterion, the IL-4 targets were categorized as ZAP-70^{Pos} or ZAP-70^{Neg}, respectively. These conclusions were validated by hierarchical clustering analysis of these sets of genes using their fold changes, resulting in segregation of patients into two clusters, one containing the ZAP-70 positive patients and the other the ZAP-70 negative patients (Figure S3). Expression of ZAP-70, CD38, and ITGA4 significantly correlated in CLL ($R = 0.512$ for ZAP-70 and CD38, $R = 0.509$ for ZAP-70 and ITGA4, and $R = 0.393$ for CD38 and ITGA4); and in general, also correlated similarly with the IL-4 targets (e.g., positive correlations with SUSD2, CABIN1, OBFC2A, SLC5A12, SLC37A3, PLD6, or negative correlations with EVI2A), though the number of IL-4 targets significantly correlated with ZAP-70 was higher than with CD38 or ITGA4 (Table S10). Analysis of the gene response to IL-4 according to cytogenetic characteristics of patients or between untreated and previously treated patients did not result in significant findings, possibly due to the low number of cases within some of the groups compared. Therefore, the gene response to IL-4 is related to the expression of CLL prognostic markers, especially ZAP-70.

Functional interpretation of the IL-4 target data sets using the MetaCore database and analysis tools

Based on upregulation of specific genes, MetaCore analysis of the CLL data set suggested activation of Wnt signaling and cell adhesion, whereas analysis of the NBC data set suggested activation of Creb signaling and angiogenesis (Fig. 4A). Analysis of the ZAP-70^{Pos} data set suggested activation of Wnt signaling, regulation of epithelial to mesenchymal transition, and cell adhesion, whereas analysis of the ZAP-70^{Neg} data set suggested activation of oxidative stress regulation, and angiogenesis (Figure 4B). Taken together, MetaCore functional analyses support that IL-4 may transduce specific pathways in CLL and NBC, and according to ZAP-70 expression in CLL.

Correlations between IL-4 targets and apoptosis

Apoptosis was studied by flow cytometry analysis of annexin V positive cells. IL-4 significantly reduced spontaneous apoptosis of CLL cells, but not of NBC cells in this time period (Figure 5A). Therefore, CLL cells were more prone to spontaneous apoptosis but also better protected by IL-4 than NBC, in agreement with previous studies [13]. Differential basal expression between CLL and NBC of apoptosis-related genes, such as Bcl2 family members, and/or changes of expression during culture, might account for the different sensitivity to spontaneous apoptosis. Within the Bcl2 family, we observed significant basal overexpression of pro-apoptotic BBC3 and BMF in CLL, but also underexpression of pro-apoptotic BCL2L11/BIM (Table S2). According to MetaCore analysis, additional 86 apoptosis-related genes had different basal levels (data not shown), being some of them more than 10-fold underexpressed in CLL (GNG11, IL6, FHL2, NGFRAP1, and ITGB3). Cell culture induced changes of an elevated number of genes that persisted in the presence of IL-4 in CLL (Table S6) and in NBC (Table S7). Of them, FOS, FOSB, DUSP1, CEBPD, and ITGB2 were more than 5-fold downregulated in both cell types, GZMA and JUN had stronger downregulation in CLL (indeed, JUN was one of the IL-4 targets (Table S3)), and MYLK,

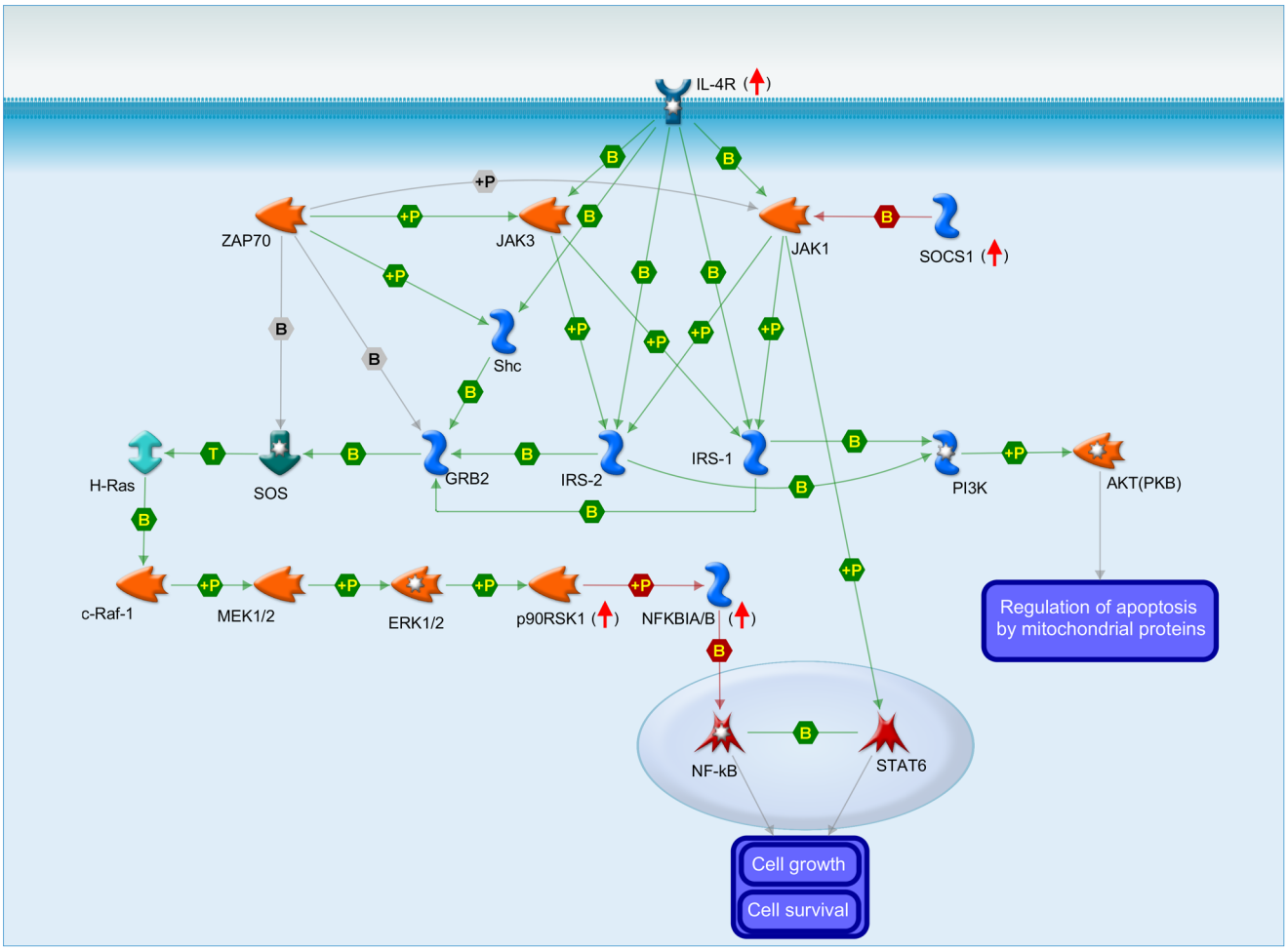

Figure 6. Map of the IL-4 signaling pathway and the potential role of ZAP-70. Adaptation of the pathway map entitled "Immune response_IL-4 – anti-apoptotic action" from MetaCore from Thomson Reuters. The map has been simplified leaving the minimal elements for activation of transcription factors essential in regulation of gene expression. ZAP-70 and its interactions with members of the pathway reported in the MetaCore database have been added to suggest its potential involvement in the pathway, and a link has been added for the reported interaction between NFκB and STAT6 [21]. Red arrows indicate that IL4R, SOCS1, RPS6AK2, and NFKBIZ are components of the pathway identified as IL-4 upregulated targets in this study. Other genes of the pathway were significantly regulated by cell culture, such as AKT3 (up) and GRB2 (down).
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PRKAR2B, SELP, FHL2, JUND, SMPD3, and CLDN5, in NBC. Within the Bcl2 family, BCLAF1 was downregulated by cell culture similarly in CLL and NBC, and BMF was upregulated by cell culture only in NBC. With regards to the differential anti-apoptotic effect of IL-4, MetaCore analysis identified, as related to apoptosis, the IL-4 upregulated targets CASP3, CCR2, CISH, GFI1, ICAM1, LNPEP, NCF2, NFKBIZ, RPS6KA2, SOCS1, and XBP1, and the IL-4 downregulated target GADD45B, in CLL (Table S3), but only CCR2, CISH, SOCS1, and XBP1, in NBC (Table S4). In addition, CAMKK1 and ESR2 were upregulated by cell culture but recovered their baseline levels by IL-4 in CLL (Table S8), and GNG4 and HRK were upregulated, and JAK2 was downregulated by cell culture, but their levels were stabilized by IL-4 in NBC (Table S9). Therefore, many genes may contribute to the increased response of CLL to IL-4. In addition, we compared the percentages of protection and the levels of change of the IL-4 targets in CLL, and found significant correlations, pointing out new potential anti-apoptotic players, of which HOMER2 and BCL6 had the highest increases (Table 2). No significant differences were observed in the levels of spontaneous apoptosis or protection by IL-4 between ZAP-70 positive and negative patients (Figure 5A), despite the fact that levels of some apoptosis-related IL-4 targets suffered significantly higher increases in ZAP-70 positive patients (SOCS1, NFKBIZ) or in ZAP-70 negative patients (LNPEP, RPS6KA2).

Effects of an NFκB activation inhibitor on the gene expression response to IL-4

To investigate the role of NFκB on the gene expression response to IL-4 in CLL, we used a quinazoline that inhibits the transactivation capacity of NFκB [35], in a ZAP-70 positive patient (CLL01). We found that the NFκB inhibitor preferentially downregulated the response of the ZAP-70^{Pos} genes, and upregulated the response of the ZAP-70^{Neg} genes (Table S10). The NFκB activation inhibitor counteracted the anti-apoptotic effect of IL-4 in CLL in a dose-dependent manner, especially in ZAP-70 positive patients at 10 μM (Figure 5A). The response of selected ZAP-70^{Pos} targets (SUSD2, and CCR2 (Figure 5B)), and ZAP-70^{Neg} targets (AUH, LY75, NFIL3, and BCL6 (Figure 5C) to IL-4 plus NFκB activation inhibitor was confirmed by qPCR in 7 ZAP-70 positive and 8 ZAP-70 negative patients. Upregulation of

the ZAP-70^{Neg} targets by the NFκB inhibitor was higher in ZAP-70 negative patients.

Discussion

The study of the changes in the GEPs induced by microenvironmental factors may help to understand the underlying mechanisms sustaining CLL pathogenesis. Despite the fact that IL-4 has been recognized as a key survival factor in CLL for a long time, the GEPs induced by IL-4 in CLL are poorly known. Here, we identified 229 well-defined genes as IL-4 targets in CLL and NBC altogether, most of which were novel IL-4 targets for CLL, B cells of diverse origin, lymphocytes, or other cell types. The previously known IL-4 targets that were also identified in our study provided a proof of validity for our microarray study. An additional validation was obtained by qPCR analysis on a significant set of genes. The introduction of two reference samples helped to define accurately the IL-4 targets by exclusion of those genes modulated by cell culture, but in general, comparison IL-4 vs Ctrl was necessary and sufficient.

In our analysis, the number of IL-4 targets was higher in CLL than in NBC. This outcome has two alternative (non-exclusive) explanations. First, that the different size of patient and control populations favors an increased detection of statistically significant targets with relatively heterogeneous response in CLL. Second, that indeed CLL have a stronger gene expression response. A substantial part of the gene response was common to CLL and NBC, qualitatively and quantitatively. However, sets of 54 and 11 genes with differential responses (most of them specific) were found in CLL and NBC, respectively. Previous studies had reported increased expression of the IL-4 receptor in CLL, at the protein [13], and the mRNA level by microarray [5], but in another study differences were not found [36]. In our study, as in the latter, no significant differences were observed between the basal levels of IL4R mRNA in CLL and NBC. The first two studies were performed with lower number of samples and/or used B cell samples of different source (tonsil). Furthermore, in our study the IL4R gene was induced similarly by IL-4 in CLL and NBC, suggesting that the differential response arises downstream of IL4R.

Correlations with useful prognostic markers may help to reveal new altered pathways and alternative therapeutic targets. We observed that two sets of IL-4 targets had changes correlated positively and negatively with the basal levels of ZAP-70. ZAP-70 has not been previously related to the IL-4 pathway, but several interactions with components of this pathway had been reported (Figure 6). Differentially regulated genes play essential roles in developmental and survival pathways, such as Wnt signaling and cell adhesion in the ZAP-70^{Pos} set (CDH1, WNT5B, WNT11). CDH1 expression was reported to be repressed epigenetically in CLL [37], and our data suggest that IL-4 overcomes this repression, especially in ZAP-70 positive patients (and also in patients expressing the adhesion protein ITGA4). These data may be related with recent evidence that ZAP-70 positive CLL cells exhibit higher adhesion capacity to stromal cells in response to CD40L+IL-4 [38]. Previous studies have detected increased levels of the anti-apoptotic proteins BCL2, using IL-4 [24], or MCL1, BCL2L1, BCL2A1, or XIAP, using the CD40L/IL-4 system [39,40], without correlation at the mRNA level, and our study using IL-4 alone did not detect significant changes of expression of their transcripts either. However, our study contributes several candidate genes for the anti-apoptotic mechanism of IL-4 in CLL, for the higher sensitivity of CLL cells to cell culture, and for the higher protective effect of IL-4 in CLL cells compared to NBC. Some of the IL-4 targets, and some of the genes whose levels were altered by culture but stabilized by IL-4, were previously related to apoptosis, and their responses were often stronger in CLL (e.g. CASP3, GFI1, ICAM1, LNPEP, NCF2, NFKBIZ, RPS6KA2, GADD45B). In addition, several IL-4 targets, some of which had not been previously related to apoptosis, correlated to the levels of cytoprotection. The most upregulated genes of this list, HOMER2 and BCL6, also had significant stronger responses in CLL (HOMER2 for comparison IL-4 vs Pre, and BCL6 for comparison IL-4 vs Ctrl, Table S3). HOMER2 belongs to a family of scaffolding proteins that prevent neuronal apoptosis through PI3K and the glutamate receptor [41], and regulate T cell activation by binding to NFAT [42], but its role in B cells remains largely unknown. BCL6 is a repressor transcription factor associated to worse prognosis in CLL [43]. The identification of these genes provides a starting point for future studies aimed at defining precisely the survival mechanism of IL-4. In contrast to the study of Coscia and co-workers [23] which reports lower sensitivity to spontaneous apoptosis of CLL cells expressing poor prognosis markers, we did not observe significant differences in sensitivity of ZAP-70 positive and negative patients, despite differential regulation of several apoptosis-related IL-4 targets (SOCS1, NFKBIZ, LNPEP, RPS6KA2). However, we did observe differential cytoprotection following treatment with IL-4 and an NFκB inhibitor (see below).

NFκB and ZAP-70 are effectors of the BCR signalling pathway. NFκB expression is induced following BCR signalling, and associates to cell survival and expression of ZAP-70 in CLL [44]. ZAP-70 enhances the BCR signalling responses in CLL [45,46]. NFκB inhibitors induce apoptosis of CLL cells, and ZAP-70 positive patients have higher NFκB activity and increased sensitivity [47,48]. We show that an NFκB inhibitor counteracted the anti-apoptotic effect of IL-4, especially in ZAP-70 positive patients, and the gene expression response of a great part of IL-4 targets, especially the ZAP-70^{Pos} targets which, therefore, would depend on NFκB for IL-4 responsiveness. However, the inhibitor potentiated the response of a collection of genes, especially the ZAP-70^{Neg} targets, suggesting a novel mechanism by which ZAP-70 and NFκB could work together to diminish responsiveness to IL-4 of this gene set. Examples of genes of both groups were confirmed by qPCR, such as CCR2, the chemokine receptor of CCL2, involved in CLL survival [49], NFIL3, a pro-survival transcription factor in B cells [50], and BCL6. Revealing the existence of this dual mechanism has been made possible by performing transcriptome-wide studies. The search for a molecular explanation, including the role played by ZAP-70, will be the goal of future studies. JAK3 has also been targeted with promising results in CLL using the specific inhibitor PF-956980 [25]. Our preliminary data indicate that PF-956980, similarly to the NFκB inhibitor, counteracts protection by IL-4, but abrogates completely the gene expression response both of ZAP-70^{Pos} and ZAP-70^{Neg} targets (data not shown), suggesting that targeting this step, which blocks STAT6 activation, fully abolishes the pathway.

In summary, the present study identifies sets of new genes that respond differentially to IL-4 in CLL depending on ZAP-70 expression and NFκB activation, contributing to the understanding of the anti-apoptotic response to IL-4 of CLL. In the context of evaluating inhibition of the IL-4 pathway as a therapeutic strategy in CLL, several steps may be targeted. Because inhibition of NFκB counteracts cytoprotection by IL-4, and is associated to an attenuated response of a set of IL-4 targets, NFκB targeting should be further explored especially in CLL patients expressing bad prognostic markers.

Supporting Information

Figure S1 Comparison of baseline GEPs of CLL and NBC. Heatmap representation of genes basally underexpressed (A) and overexpressed (B) in CLL compared to NBC, above a cut-off value of 3-fold change, and p values of less than 0.001, at time zero after purification of B cells. In the event that several probes represent the same gene, only one is shown. The relative level of gene expression is depicted according to the shown color scale.
(TIF)

Figure S2 Validation of microarray analysis by qPCR. Pearson correlation analysis between microarray and qPCR for 16 IL-4 targets. Microarray data were expressed as log fold change ratios, and qPCR data as $-\Delta\Delta$Ct ratios. The CLL and NBC samples are represented together. Correlation coefficients (r) and p-values are indicated. The IL-4 targets were ordered alphabetically.
(TIF)

Figure S3 Hierarchical clustering analysis for IL-4 upregulated targets correlated with ZAP70. (A) Hierarchical clustering analysis using the $ZAP70^{Pos}$ IL-4 targets. (B) Hierarchical clustering analysis using the $ZAP70^{Neg}$ IL-4 targets. In order to make groups of similar size, we selected, among the 23 patients studied by microarray, the 5 patients with the lowest levels of ZAP70, the 5 patients with the highest levels of ZAP70 and other 6 patients with intermediate values. Both analyses were able to separate CLL into two clusters containing the 5 positive and 5 negative patients. In the event that several probes represent the same gene, only one is shown. Fold changes for the IL-4 targets, or relative levels for basal expression of ZAP70 by microarray, are depicted according to the shown $\log_2$ color scale.
(TIF)

Table S1 Characteristics of CLL patients.
(XLS)

Table S2 Differential basal gene expression levels between CLL and NBC.
(XLS)

Table S3 IL-4 targets in CLL and comparison of their responses with those in NBC.
(XLS)

Table S4 IL-4 targets in NBC and comparison of their responses with those in CLL.
(XLS)

Table S5 Novel and previously known IL-4 targets in diverse cell types (with references) among the IL-4 targets identified in CLL and NBC in the present study.
(XLS)

Table S6 Gene expression changes induced by culture not counteracted by IL-4 in CLL.
(XLS)

Table S7 Gene expression changes induced by culture not counteracted by IL-4 in NBC.
(XLS)

Table S8 Gene expression changes induced by culture counteracted by IL-4 in CLL.
(XLS)

Table S9 Genes expression changes induced by culture counteracted by IL-4 in NBC.
(XLS)

Table S10 Effects of an NFκB activation inhibitor on the response of the IL-4 upregulated targets in a CLL patient (CLL01, Zap70 positive).
(XLS)

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Author Contributions

Conceived and designed the experiments: NRL MJAG AP. Performed the experiments: NRL MJAG SSR NMB AM AP. Analyzed the data: NRL MJAG AP. Contributed reagents/materials/analysis tools: JGE CF MCGG JMM MRAL. Wrote the paper: AP.

References

1. Chiorazzi N (2012) Implications of new prognostic markers in chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program 2012: 76–87.
2. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, et al. (2001) Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med 194: 1625–1638.
3. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, et al. (2001) Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 194: 1639–1647.
4. Stratowa C, Löffler G, Lichter P, Stilgenbauer S, Haberl P, et al. (2001) CDNA microarray gene expression analysis of B-cell chronic lymphocytic leukemia proposes potential new prognostic markers involved in lymphocyte trafficking. Int J Cancer 91: 474–480.
5. Zheng Z, Venkatapathy S, Rao G, Harrington CA (2002) Expression profiling of B cell chronic lymphocytic leukemia suggests deficient CD1-mediated immunity, polarized cytokine response, altered adhesion and increased intracellular protein transport and processing of leukemic cells. Leukemia 16: 2429–2437.
6. Jelinek DF, Tschumper RC, Stolovitzky GA, Iturria SJ, Tu Y, et al. (2003) Identification of a global gene expression signature of B-chronic lymphocytic leukemia. Mol Cancer Res 1: 346–361.
7. Wang J, Coombes KR, Highsmith WE, Keating MJ, Abruzzo LV (2004) Differences in gene expression between B-cell chronic lymphocytic leukemia and normal B cells: a meta-analysis of three microarray studies. Bioinformatics 20: 3166–3178.
8. Dürig J, Nückel H, Hüttmann A, Kruse E, Hölter T, et al. (2003) Expression of ribosomal and translation-associated genes is correlated with a favorable clinical course in chronic lymphocytic leukemia. Blood 101: 2748–2755.
9. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, et al. (2003) ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 101: 4944–4951.
10. Stankovic T, Hubank M, Cronin D, Stewart GS, Fletcher D, et al. (2004) Microarray analysis reveals that TP53- and ATM-mutant B-CLLs share a defect in activating proapoptotic responses after DNA damage but are distinguished by major differences in activating prosurvival responses. Blood 103: 291–300.
11. Rodríguez A, Villuendas R, Yáñez L, Gómez ME, Díaz R, et al. (2007) Molecular heterogeneity in chronic lymphocytic leukemia is dependent on BCR signaling: clinical correlation. Leukemia 21: 1984–1991.
12. Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, et al. (2012) Cellular origin and pathophysiology of chronic lymphocytic leukemia. J Exp Med 209: 2183–2198.
13. Douglas RS, Capocasale RJ, Lamb RJ, Nowell PC, Moore JS (1997) Chronic lymphocytic leukemia B cells are resistant to the apoptotic effects of Transforming Growth Factor-β. Blood 89: 941–947.
14. Burger JA, Montserrat E (2013) Coming full circle: 70 years of chronic lymphocytic leukemia cell redistribution, from glucocorticoids to inhibitors of B-cell receptor signaling. Blood 121: 1501–1509.
15. Okada H, Banchereau J, Lotze MT (2003) Interleukin-4. In Thompson AW, Lotze MT, editors. The Cytokine Handbook, Vol. I. London: Academic Press. pp 227–262.
16. Zamorano J, Mora AL, Boothby M, Keegan AD (2001) NF-kappa B activation plays an important role in the IL-4-induced protection from apoptosis. Int Immunol 13: 1479–1487.

17. Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ (2000) Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol 164: 2200–2206.
18. Cuní S, Pérez-Aciego P, Pérez-Chacón G, Vargas JA, Sánchez A, et al. (2004) A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. Leukemia 18: 1391–1400.
19. Thieu VT, Nguyen ET, McCarthy BP, Bruns HA, Kapur R, et al. (2007) IL-4-stimulated NF-kappaB activity is required for Stat6 DNA binding. J Leukoc Biol 82: 370–379.
20. Bhattacharya N, Reichenzeller M, Caudron-Herger M, Haebe S, Brady N, et al. (2014) Loss of cooperativity of secreted CD40L and increased dose-response to IL4 on CLL cell viability correlates with enhanced activation of NF-kB and STAT6. Int J Cancer. doi: 10.1002/ijc.28974. [Epub ahead of print].
21. Shen CH, Stavnezer J (1998) Interaction of stat6 and NF-kappaB: direct association and synergistic activation of interleukin-4-induced transcription. Mol Cell Biol 18: 3395–3404.
22. Messner B, Stütz AM, Albrecht B, Peiritsch S, Woisetschläger M (1997) Cooperation of binding sites for STAT6 and NF kappa B/rel in the IL-4-induced up-regulation of the human IgE germline promoter. J Immunol 159: 3330–3337.
23. Coscia M, Pantaleoni F, Riganti C, Vitale C, Rigoni M, et al. (2011) IGHV unmutated CLL B cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. Leukemia 25: 828–837.
24. Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G, et al. (1992) Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. J Exp Med 176: 1319–1326.
25. Steele AJ, Prentice AG, Cwynarski K, Hoffbrand AV, Hart SM, et al. (2010) The JAK3-selective inhibitor PF-956980 reverses the resistance to cytotoxic agents induced by interleukin-4 treatment of chronic lymphocytic leukemia cells: potential for reversal of cytoprotection by the microenvironment. Blood 116: 4569–4577.
26. Mainou-Fowler T, Proctor SJ, Miller S, Dickinson AM (2001) Expression and production of interleukin 4 in B-cell chronic lymphocytic leukaemia. Leuk Lymphoma 42: 689–698.
27. Pangault C, Amé-Thomas P, Ruminy P, Rossille D, Caron G, et al. (2010) Follicular lymphoma cell niche: identification of a preeminent IL-4-dependent T(FH)–B cell axis. Leukemia 24: 2080–2089.
28. Schroder AJ, Pavlidis P, Arimura A, Capece D, Rothman PB (2002) Cutting edge: STAT6 serves as a positive and negative regulator of gene expression in IL-4-stimulated B lymphocytes. J Immunol 168: 996–1000.
29. Lu X, Nechushtan H, Ding F, Rosado MF, Singal R, et al. (2005) Distinct IL-4-induced gene expression, proliferation, and intracellular signaling in germinal center B-cell-like and activated B-cell-like diffuse large-cell lymphomas. Blood 105: 2924–2932.
30. Stamatopoulos B, Meuleman N, De Bruyn C, Pieters K, Anthoine G, et al. (2010) A molecular score by quantitative PCR as a new prognostic tool at diagnosis for chronic lymphocytic leukemia patients. PLoS ONE 5: 12780.
31. Early SB, Barekzi E, Negri J, Hise K, Borish L, et al. (2007) Concordant modulation of cysteinyl leukotriene receptor expression by IL-4 and IFN-gamma on peripheral immune cells. Am J Respir Cell Mol Biol 36: 715–720.
32. Finkelman FD, Katona IM, Urban JF Jr, Snapper CM, Ohara J, et al. (1986) Suppression of in vivo polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor 1. Proc Natl Acad Sci USA 83: 9675–9678.
33. Kashiwada M, Levy DM, McKeag L, Murray K, Schröder AJ, et al. (2010) IL-4-induced transcription factor NFIL3/E4BP4 controls IgE class switching. Proc Natl Acad Sci USA 107: 821–826.
34. Alcaraz-García MJ, Ruiz-Lafuente N, Sebastián-Ruiz S, Majado MJ, González-García C, et al. (2011) Human and mouse DOCK10 splicing isoforms with alternative first coding exon usage are differentially expressed in T and B lymphocytes. Hum Immunol 72: 531–537.
35. Tobe M, Isobe Y, Tomizawa H, Nagasaki T, Takahashi H, et al. (2003) A novel structural class of potent inhibitors of NF-kappa B activation: structure-activity relationships and biological effects of 6-aminoquinazoline derivatives. Bioorg Med Chem 11: 3869–3878.
36. Kaminski A, Demaine A, Prentice A (1998) Cytoplasmic interleukin-4 (IL-4) and surface IL-4 receptor expression in patients with B-cell lymphocytic leukemia. Blood 92: 2188–2189.
37. Moskalev EA, Luckert K, Vorobjev IA, Mastitsky SE, Gladkikh AA, et al. (2012) Concurrent epigenetic silencing of wnt/β-catenin pathway inhibitor genes in B cell chronic lymphocytic leukaemia. BMC Cancer 2012; 12: 213.
38. Lafarge ST, Johnston JB, Gibson SB, Marshall AJ (2014) Adhesion of ZAP-70+ chronic lymphocytic leukemia cells to stromal cells is enhanced by cytokines and blocked by inhibitors of the PI3-kinase pathway. Leuk Res 38: 109–115.
39. Willimott S, Baou M, Naresh K, Wagner SD (2007) CD154 induces a switch in pro-survival Bcl-2 family members in chronic lymphocytic leukaemia. Br J Haematol 138: 721–732.
40. Cosimo E, McCaig AM, Carter-Brzezinski LJM, Wheadon H, Leach MT, et al. (2013) Inhibition of NF-kB-mediated signaling by the cyclin-dependent kinase inhibitor CR8 overcomes prosurvival stimuli to induce apoptosis in chronic lymphocytic leukemia cells. Clin Cancer Res 19: 2393–2405.
41. Rong R, Ahn JY, Huang H, Nagata E, Kalman D, et al. (2003) PI3 kinase enhancer-Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis. Nat Neurosci 6: 1153–1161.
42. Huang GN, Huso DL, Bouyain S, Tu J, McCorkell KA, et al. (2008) NFAT binding and regulation of T cell activation by the cytoplasmic scaffolding Homer proteins. Science 319: 476–481.
43. Jantus Lewintre E, Reinoso Martín C, García Ballesteros C, Pendas J, Benet Campos C, et al. (2009) BCL6: somatic mutations and expression in early-stage chronic lymphocytic leukemia. Leuk Lymphoma 50: 773–780.
44. López-Guerra M, Roué G, Pérez-Galán P, Alonso R, Villamor N, et al. (2009) p65 activity and ZAP-70 status predict the sensitivity of chronic lymphocytic leukemia cells to the selective IkappaB kinase inhibitor BMS-345541. Clin Cancer Res 15: 2767–2776.
45. Chen L, Huynh L, Apgar J, Tang L, Rassenti L, et al. (2008) ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. Blood 111: 2685–2692.
46. Calpe E, Codony C, Baptista MJ, Abrisqueta P, Carpio C, et al. (2011) ZAP-70 enhances migration of malignant B lymphocytes toward CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation. Blood 118: 4401–4410.
47. Pickering BM, de Mel S, Lee M, Howell M, Habens F, et al. (2007) Pharmacological inhibitors of NF-kappaB accelerate apoptosis in chronic lymphocytic leukaemia cells. Oncogene 26: 1166–1177.
48. Hewamana S, Alghazal S, Lin TT, Clement M, Jenkins C, et al. (2008) The NF-kappaB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. Blood 111: 4681–4689.
49. Burgess M, Cheung C, Chambers L, Ravindranath K, Minhas G, et al. (2012) CCL2 and CXCL2 enhance survival of primary chronic lymphocytic leukemia cells in vitro. Leuk Lymphoma 53: 1988–1998.
50. Ikushima S, Inukai T, Inaba T, Nimer SD, Cleveland JL, et al. (1997) Pivotal role for the NFIL3/E4BP4 transcription factor in interleukin 3-mediated survival of pro-B lymphocytes. Proc Natl Acad Sci U S A 94: 2609–2614.

Figure S1

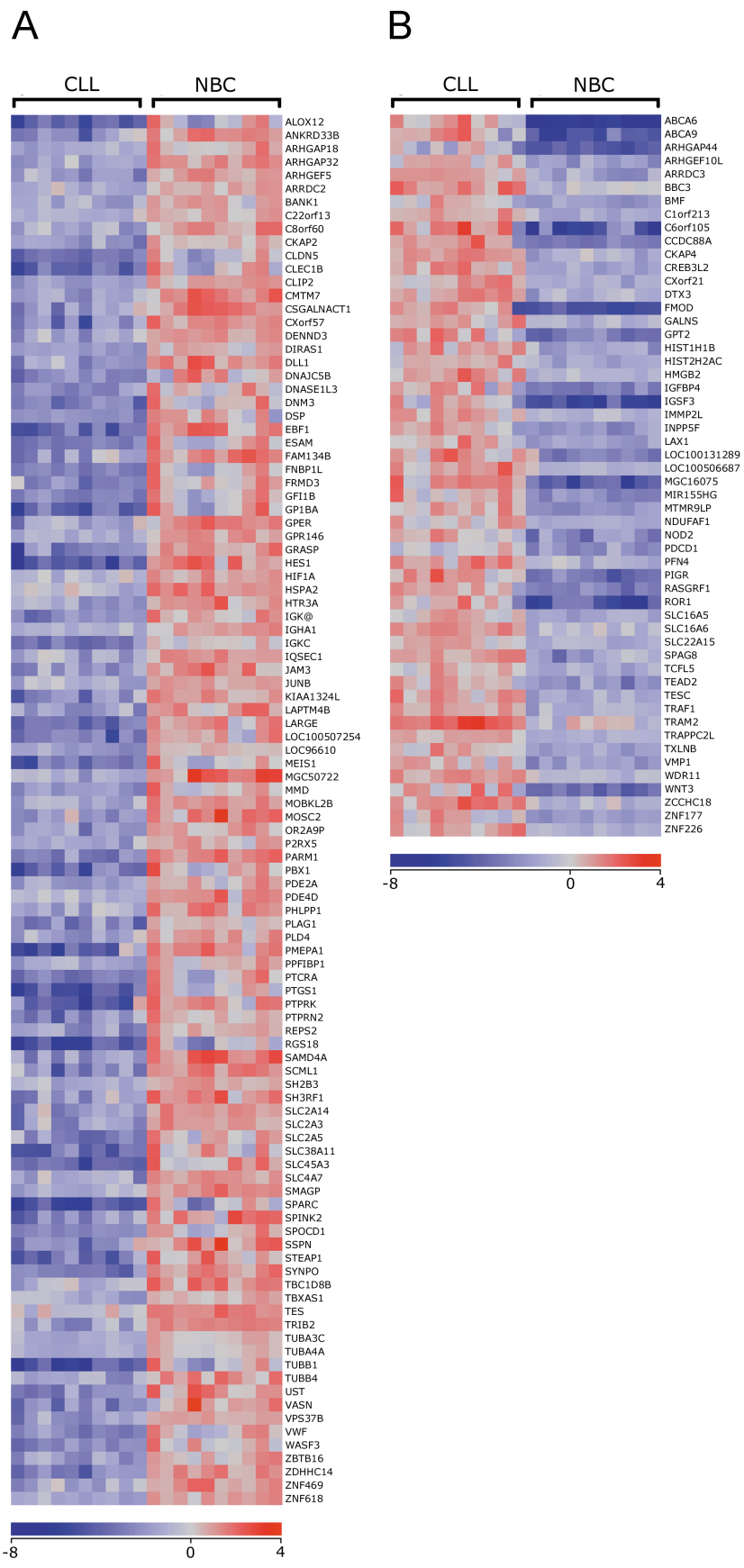
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Figure S2

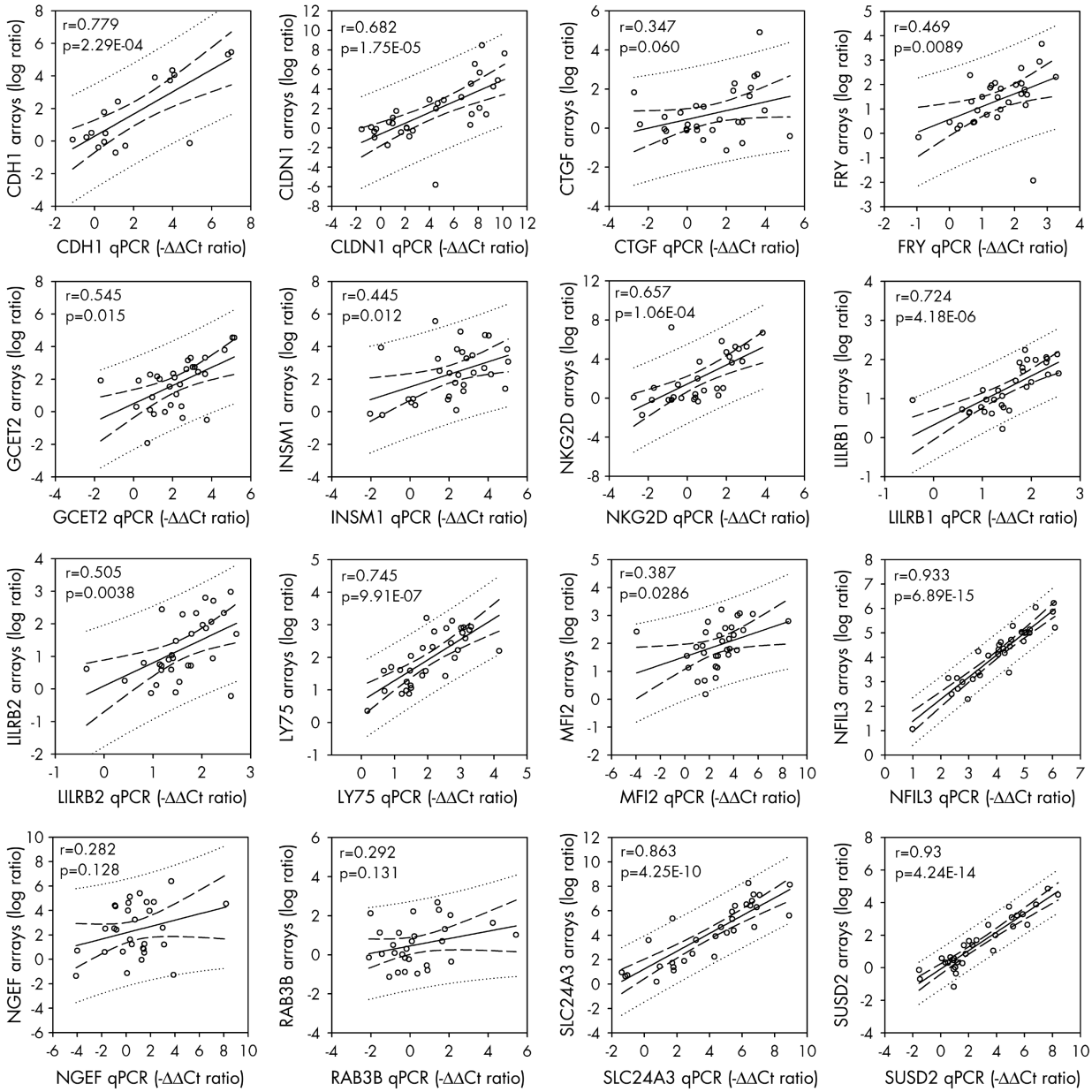
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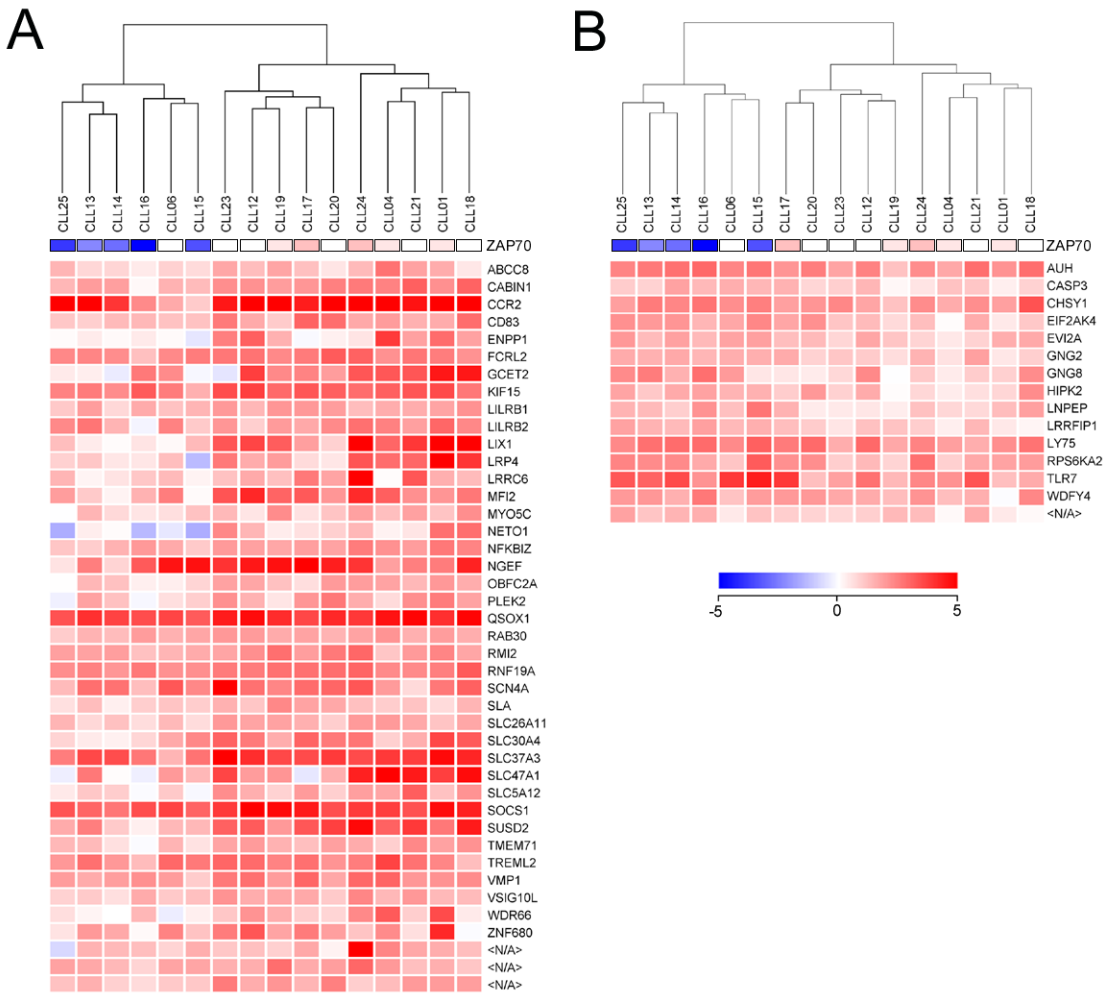
Figure S3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RESEARCH ARTICLE

IL-4 Up-Regulates MiR-21 and the MiRNAs Hosted in the CLCN5 Gene in Chronic Lymphocytic Leukemia

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Abstract

Interleukin 4 (IL-4) induces B-cell differentiation and survival of chronic lymphocytic leukemia (CLL) cells. MicroRNAs (miRNAs) regulate mRNA and protein expression, and several miRNAs, deregulated in CLL, might play roles as oncogenes or tumor suppressors. We have studied the miRNA profile of CLL, and its response to IL-4, by oligonucleotide microarrays, resulting in the detection of a set of 129 mature miRNAs consistently expressed in CLL, which included 41 differentially expressed compared to normal B cells (NBC), and 6 significantly underexpressed in ZAP-70 positive patients. IL-4 stimulation brought about up-regulation of the 5p and 3p mature variants of the miR-21 gene, which maps immediately downstream to the VMP1 gene, and of the mature forms generated from the miR-362 (3p and 5p), miR-500a (3p), miR-502 (3p), and miR-532 (3p and 5p) genes, which map within the third intron of the CLCN5 gene. Both genes are in turn regulated by IL-4, suggesting that these miRNAs were regulated by IL-4 as passengers from their carrier genes. Their levels of up-regulation by IL-4 significantly correlated with cytoprotection. MiR-21 has been reported to be leukemogenic, associated to bad prognosis in CLL, and the miRNA more frequently overexpressed in human cancer. Up-regulation by IL-4 of miR-21 and the miRNAs hosted in the CLCN5 locus may contribute to evasion of apoptosis of CLL cells. These findings indicate that the IL-4 pathway and the miRNAs induced by IL-4 are promising targets for the development of novel therapies in CLL.

Introduction

The interleukin-4 (IL-4) pathway leads to maturation of B-cell precursors into immunoglobulin-secreting cells and antigen presenting cells, proliferation of activated B cells, and induction

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of isotype switching toward IgE [1]. IL-4 protects chronic lymphocytic leukemia (CLL) cells from spontaneous apoptosis or killing with DNA damaging agents [2–5]. CLL is a B-cell malignant disease most prevalent in the elderly, characterized by surface expression of the CD5 and CD23 markers, and a heterogeneous clinical course, with patients divided between those that never progress to late stages of the disease, and those that progress and require therapy. Prognostic markers such as IGVH status and ZAP-70 and CD38 expression levels are useful to evaluate the risk of progression [6]. Through its cytoprotective effect, the IL-4 pathway may sustain evasion of apoptosis of CLL cells, thereby contributing to leukemogenesis.

Binding of IL-4 to its surface receptor (IL-4R) induces phosphorylation of JAK1 and JAK3. JAK1 phosphorylates STAT6 which homodimerizes and enters the nucleus to regulate gene expression. JAK1 and JAK3 lead to anti-apoptotic signaling through PI3K/AKT and the mitochondrial pathway, and through the Ras/MAPK pathway and NFκB activation [7–11]. Recently, we have reported gene expression changes induced by IL-4 in CLL [12], but little is known about the response to IL-4 of microRNAs (miRNAs), an essential class of gene expression regulators.

Mature miRNAs are non-coding RNAs of 19–25 nucleotides in length, generated by processing of miRNA gene transcripts called pri-miRNAs. Based on their genomic localization, miRNAs can be divided into two main classes: intergenic, that constitute independent transcription units, and intragenic, located inside another gene and produced as part of the host gene mRNA [13]. The pri-miRNAs are capped and polyadenylated, then cropped by the Microprocessor complex, and the resulting stem-loop intermediate, called pre-miRNA, is exported to the cytoplasm. The pre-miRNA is further cleaved to generate miRNA duplexes in the RNA-induced silencing complex (RISC), where one or the other strand (5p or 3p) is degraded. The remaining strand, which constitutes the mature miRNA, is retained in the RISC and will target mRNAs by base-pairing to complete or partially complementary sites on the target mRNAs, usually located at the 3' untranslated regions. As a consequence, gene expression is negatively regulated through mRNA degradation or, more commonly, translational repression. A single miRNA could repress expression of up to several hundred genes.

Deregulation of miRNAs has been implicated in human oncogenesis. In CLL, several miRNAs have been recurrently found overexpressed compared to normal B cells (NBC), such as miR-155 [14–19], miR-150 [14,16,19], miR-101 [14,18,19], miR-21 [14,18], miR-29a [18,19], or miR-29c [16,19], or underexpressed, such as miR-181a, miR-181b [15,18,19], and miR-223 [15,16,19]. CLL patients characterized by 13q14 or 17p deletions usually underexpress miR-15a [20,21] or miR-34a [16,22–25], located at the respective deleted regions, compared to other cytogenetic subtypes. Diverse animal models have illustrated the oncogenic potential of several miRNAs, including miR-155, miR-21, miR-29a, or the miR-17~92 cluster [26–29], and the tumor suppressor potential of others, such as the miR-15a/16-1 cluster [30,31]. MiRNA signatures frequently include higher expression of miR15a, miR-16, or miR-23b in patients expressing markers of worse prognosis, such as ZAP-70 [15,24,32,33]. In ZAP-70 negative patients miR-29a, miR-29b, miR-29c and miR-223 often show higher expression levels [15,16,19,32,33].

Here we report the identification of miRNAs regulated by IL-4 in CLL. MiR-21, miR-362, miR-500a, miR-502, and miR-532 were induced by IL-4, likely as a consequence of up-regulation of their respective host genes, vacuole membrane protein 1 (VMP1), and chloride channel, voltage sensitive 5 (CLCN5). MiR-21 is the most frequently overexpressed miRNA in human cancer, and could be involved in evasion of apoptosis and resistance to chemotherapy in CLL. IL-4 could exert its anti-apoptotic function through up-regulation of miR-21 and the microRNAs hosted in the CLCN5 gene. These findings may be useful in the development of therapeutic strategies targeting the IL-4 pathway in CLL.

Methods

Sample collection

Peripheral blood samples from 16 chronic lymphocytic leukemia (CLL) patients and 3 controls with normal lymphopoiesis were obtained. The study was approved by the Review Board of Hospital Clínico Universitario Virgen de la Arrixaca, and the participants provided their written informed consent. All the patients had leukocytosis and did not receive treatment in the last 3 months prior to sample collection (S1 Table).

Cell isolation

Samples were processed to isolate the B cells by negative selection procedures which were based on cocktails containing CD2, CD16, CD36 and CD235a antibodies for depletion of T cells, NK cells, monocytes, macrophages, and erythrocytes. The RossetteSep Human B Cell Enrichment Cocktail kit (StemCell Technologies, Vancouver, Canada) was suitable for CLL samples, since these are rich in malignant B cells. This method is expensive but definitely worthwhile for cell populations present at relatively high levels. Small volumes of peripheral blood (10 mL) were collected from patients, and B cell isolation was directly performed during Ficoll 1.077 g/mL centrifugation, following the manufacturer's instructions. However, due to the low content of B cells in the peripheral blood of normal subjects (usually less than 10% of the lymphocytes), larger volumes of peripheral blood were collected (500 mL). Thus, the use of the RossetteSep kit was not economically viable for normal samples. In addition, harvest of the fine, barely visible B cell layer formed at the interphase between diluted plasma and Ficoll is usually inefficient. An alternative method, the Dynabeads Untouched Human B cells kit (Invitrogen, Carlsbad, CA) was chosen for isolating NBC. Despite being more time consuming, this method is more efficient and less expensive. PBMC concentrates were obtained by centrifugation over Ficoll, and NBC isolated using the kit, following the manufacturer's instructions. Enrichment was determined by labelling with CD19-FITC, CD3-PE, and CD5-PE-Cy7 (BD Biosciences), followed by acquisition in a FACScalibur flow cytometer (BD Biosciences), and analysis using the CellQuest software. Purity of CD19+ cells was 93.5±1.41% (mean±s.e.m.) in NBC and 97.43±0.62% in CLL, including 0.33±0.14% of CD19+CD5- potential normal B cells within the CLL fractions (range 0–1.05%). The percentage of ZAP-70 positive cells within the CD19+CD5+ fraction of CLL was determined from aliquots of peripheral blood subjected to red cell lysis, permeabilization with the Cytofix/Cytoperm kit, and labelling with CD19-FITC, ZAP-70-PE, CD5-PE-Cy7, and CD3-APC (BD Biosciences).

Cell culture and determination of apoptosis

Following purification, three fractions of the purified CLL and NBC were processed: a) at time zero ("Pre"); b) after being cultured for 18 hours in RPMI-1640 medium supplemented with 10% fetal calf serum (Cambrex, East Rutherford, NJ) ("Ctrl"); and c) as b, but with adding 10 ng/mL of human recombinant IL-4 (BD Biosciences, San Diego, CA) ("IL-4"). Apoptosis of the cultured cells was determined by dual labelling with annexin V and propidium iodide (BD Biosciences), and flow cytometry analysis.

RNA isolation

Total RNA, including RNA from approximately 18 nucleotides upwards, was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). RNA samples were quantitated on a NanoDrop 2000 (Thermo Fisher Scientific, Whaltham, MA). RNA quality was examined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Nano Kit.

Only samples with R.I.N. (RNA Integrity Number) >7.0 were further studied. Though the RNA 6000 Kit allowed to detect the presence of small RNA, this fraction was specifically analysed using the Small RNA Kit.

Microarray analysis

From each RNA sample, 100 ng were labeled with cyanine 3-pCp (Cy3-pCp) using Agilent miRNA Complete Labeling and Hyb Kits, according to the manufacturer's protocol. The labeled miRNAs were hybridized onto Human miRNA Microarray Kit (V3, 8 × 15k) targeting 866 human and 89 human viral miRNAs. After hybridization, the microarray slides were washed and scanned in an Agilent G2565CA DNA Microarray Scanner. Images were analyzed with the Agilent Feature Extraction software. The automatically generated datasets were statistically analyzed and visualized using the GeneSpring GX software (Agilent). The miRNAs regulated by IL-4 were identified using the one-way ANOVA test ($p<0.05$) between samples Pre, Ctrl, and IL-4, with post hoc Tukey HSD analysis. The computation of p-values was performed using the Benjamini-Hochberg FDR correction. Those entities that passed the analysis with increases or decreases above 2-fold for comparisons IL-4 vs Pre, and IL-4 vs Ctrl concurrently and with the same direction of change were considered miRNAs regulated by IL-4. The same analyses were performed for comparisons between condition Pre vs all the others, and between condition Ctrl vs all the others. Our initial analysis performed with the first 8 patients showed that changes identified for comparison IL-4 vs all the others coincided with those identified for comparison IL-4 vs Ctrl using the Student's t test. Though significant changes for comparison between condition Pre vs all the others were also identified (induced by cell culture), our main purpose was to identify miRNAs regulated by IL-4. For this reason, Pre sample was judged as dispensable from that time, and was not included in subsequent patients. Samples Ctrl and IL-4 from patient CLL03 did not pass the quality controls. Thus, our final analysis included 8 Pre, 15 Ctrl, and 15 IL-4 samples from 16 patients (S1 Table) and 3 paired Ctrl and IL-4 NBC samples. Datasets were deposited at the Gene Expression Omnibus database under accession number GSE62137. Hierarchical clustering analysis was performed using the Ward's linkage method on euclidean distances. Fold changes of miRNAs regulated by IL-4 were compared between patients by Pearson correlation analysis using SigmaStat.

Quantitative PCR (qPCR)

For miRNA expression, RNA samples were retrotranscribed with the miScript II RT Kit (Qiagen) using the miScript HiSpec buffer for mature miRNA detection only, followed by qPCR with the miScript SYBR Green PCR Kit (Qiagen) in an ABI Prism 7000 Sequence Detection System, using the miScript primer assays (Qiagen) for miR-21-3p, miR-362-3p, miR-362-5p, miR-500a-3p, miR-502-3p, miR-532, miR-532-3p, and RNU6-6P, the latter used as reference for normalization (cat. nos. MS00009086, MS00009562, MS00009569, MS00031920, MS00031927, MS00010052, MS00004571, and MS00033740, respectively). For mRNA expression, RNA samples were retrotranscribed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), followed by qPCR with the SYBR Premix Ex Taq (Takara Bio, Mountain View, CA), using QuantiTect primer assays (Qiagen) for VMP1, CLCN5, ZAP-70, and GAPDH, the two latter used as a validation of flow cytometry data [34], and as reference for normalization, respectively (cat. nos. QT00066241, QT00998683, QT00209251, and QT01192646, respectively). All these procedures were performed following the manufacturer's instructions. Measures were performed in duplicate. Analysis with the 7000 System SDS software provided the cycle threshold (Ct) values. The Ct values for RNU6-6P or GAPDH were subtracted from the Ct values for the miRNAs and mRNAs, respectively, resulting in the ΔCt values. As RNA samples

include the mRNA and miRNA fractions, GAPDH was also used for miRNA normalization. Next, the average ΔCt value for the Ctrl samples was subtracted from all the ΔCt values, resulting in the ΔΔCt values. Finally, the relative expression values, expressed as fold change compared to the average Ctrl samples, were generated using the formula $2^{-\Delta\Delta Ct}$. Individual fold changes for IL-4 samples compared to Pre and Ctrl samples were calculated as the ratios between their respective relative expression values.

Semiquantitative RT-PCR

The levels of expression of VMP1 were measured using the GeneAmp RNA PCR Core Kit in a GeneAmp PCR System 9700 (both from Thermo Fisher Scientific), following the manufacturer's instructions. First, RNA samples were retrotranscribed using random hexamers. Then, VMP1 sequences were amplified from the resulting RNA/cDNA hybrids using as primers the 5'-TTGTCCAGATGAAGAGGGCA-3' and 5'-TCAAACATCCAGGACAACCAGT-3' oligonucleotides, that map at exon 6 and exon 12, respectively, and 30 cycles of PCR. As reference, GAPDH sequences were amplified using the 5'-TCATGACCACAGTCCATGCC- 3' and 5'-CATGAGGTCCACCACCCTGT-3' oligonucleotides and 23 cycles of PCR. The amplified fragments were visualized following electrophoresis in agarose gels containing SYBR Safe stain (Thermo Fisher Scientific).

Results

MiRNA expression profile of CLL

To define the set of miRNAs significantly expressed in CLL, we examined the cumulative distributions of mature miRNAs according to their signal intensity values in the arrays performed from the 8 Pre, 15 Ctrl, and 15 IL-4 CLL samples, which described an abrupt rise between the intensity values of −9 and −7, then a plateau up to −4, and a slow increase between −3 and 9 (S1 Fig). The cumulative distribution for the 3 Ctrl and 3 IL-4 NBC samples followed the same description with slight differences (S1 Fig). The absence of miRNAs significantly expressed between intensity levels of −7 and −4 led us to choose −4 as a positivity cut-off, which defined 129 and 149 miRNAs as significantly expressed in CLL (S2 Table) and NBC (S3 Table), respectively. Both lists share similarities, including the same top 10 miRNAs: miR-150-5p, miR-142-3p, miR-29a-3p, miR-21-5p, miR-16-5p, let-7g-5p, miR-29b-3p, let-7f-5p, miR-29c-3p, and let-7a-5p. However, 41 miRNAs were differentially expressed between CLL and NBC according to the Student t test (cut-off 2-fold, $p<0.05$), being 29 overexpressed in CLL, including miR-150-5p, miR-29a-3p, miR-29b-3p, let-7a-5p, miR-26a-5p, miR-451a, miR-155-5p, miR-101-3p, miR-28-5p, miR-144-5p, miR-486-5p, or miR-486-3p, and 12 underexpressed, including miR-181a-5p, miR-222-3p, miR-126-3p, miR-365a-3p, miR-181b-5p, miR-199a-3p, or miR-582-5p (Table 1). Hierarchical clustering analysis using conditions Pre, Ctrl, and IL-4, and the 129 miRNAs expressed in CLL, tended to segregate samples Pre from samples Ctrl and IL-4, and within the two latter, ZAP-70 positive from ZAP-70 negative (Fig 1). Moreover, comparison between ZAP-70 positive (n = 11) and negative (n = 5) patients by the Student t test ($p<0.05$) using the mean values of all the available samples from each patient, revealed a set of 6 miRNAs significantly underexpressed in ZAP-70 positive patients, with differences higher than 2-fold for miR-146b-5p, miR-210-3p, and miR-29c-5p, and higher than 1.5-fold for the ones with the highest levels of expression, miR-29c-3p and miR-30b-5p (Table 2). Comparisons between cytogenetic groups or untreated and previously treated patients were not performed because we did not have sufficient data in the cohorts. Clustering analysis also showed that expression was relatively stable across patients and conditions for most miRNAs. In addition, miRNAs expressed at similar levels grouped together, and the resulting miRNA sets were

Table 1. MiRNAs diferentially expressed in CLL and NBC.

Mature miRNA ID	CLL*	NBC*	Fold change†	p value§
Overexpressed in CLL				
miR-150-5p	281.7	94.86	2.97	4.95E-03
miR-29a-3p	123.1	45.21	2.72	3.83E-04
miR-29b-3p	74.97	30.09	2.49	8.96E-04
let-7a-5p	62.11	29.72	2.09	4.64E-03
miR-26a-5p	44.06	17.78	2.48	7.87E-04
miR-451a	32.22	0.408	79.01	3.07E-06
miR-155-5p	26.28	6.397	4.11	1.11E-03
miR-101-3p	15.98	6.720	2.38	3.08E-03
miR-28-5p	3.593	0.400	8.99	4.98E-04
miR-140-5p	3.496	1.558	2.24	6.81E-05
let-7c-5p	3.280	1.519	2.16	4.84E-02
miR-374a-5p	2.422	0.538	4.51	8.46E-03
miR-320b	1.928	0.523	3.69	1.61E-02
miR-590-5p	1.759	0.320	5.50	2.66E-03
miR-34a-5p	1.267	0.458	2.77	2.04E-02
miR-195-5p	1.098	0.368	2.98	4.82E-02
miR-186-5p	1.088	0.315	3.45	2.88E-02
miR-144-3p	0.829	0.002	437.1	8.63E-06
miR-361-5p	0.757	0.233	3.26	2.50E-02
miR-23b-3p	0.754	0.198	3.81	3.56E-02
miR-30e-3p	0.713	0.205	3.47	2.87E-02
miR-192-5p	0.631	0.143	4.40	6.54E-03
miR-98-5p	0.622	0.182	3.41	2.13E-02
miR-486-5p	0.583	0.013	43.71	1.26E-04
miR-374b-5p	0.526	0.079	6.63	4.08E-03
miR-486-3p	0.276	0.013	21.7	2.30E-05
miR-128-3p	0.232	0.050	4.67	3.34E-02
miR-210-3p	0.202	0.022	9.32	1.22E-02
miR-141-3p	0.078	0.010	7.76	1.57E-02
Underexpressed in CLL				
miR-181a-5p	0.004	1.811	-417	3.10E-07
miR-222-3p	0.247	0.978	-3.96	2.15E-02
miR-126-3p	0.007	0.343	-50.7	2.80E-03
miR-365a-3p	0.017	0.318	-18.3	4.26E-02
miR-181b-5p	0.009	0.212	-24.9	1.94E-02
miR-199a-3p	0.007	0.198	-26.7	3.42E-03
miR-582-5p	0.002	0.195	-78.9	6.29E-09
miR-130a-3p	0.005	0.114	-21.6	1.79E-02
miR-155-3p	0.017	0.106	-6.12	4.86E-02
miR-501-5p	0.007	0.053	-8.11	9.73E-03
miR-132-3p	0.003	0.043	-13.4	1.25E-03
miR-30a-5p	0.006	0.041	-6.86	3.32E-02

*Relative mean values of 16 CLL and 3 NBC cases, whose values were calculated as the mean of all available samples (Pre, Ctrl, IL-4), except in the case of miRNAs modulated by IL-4, where these samples were excluded.

†CLL vs NBC.

§Student t test CLL vs NBC.

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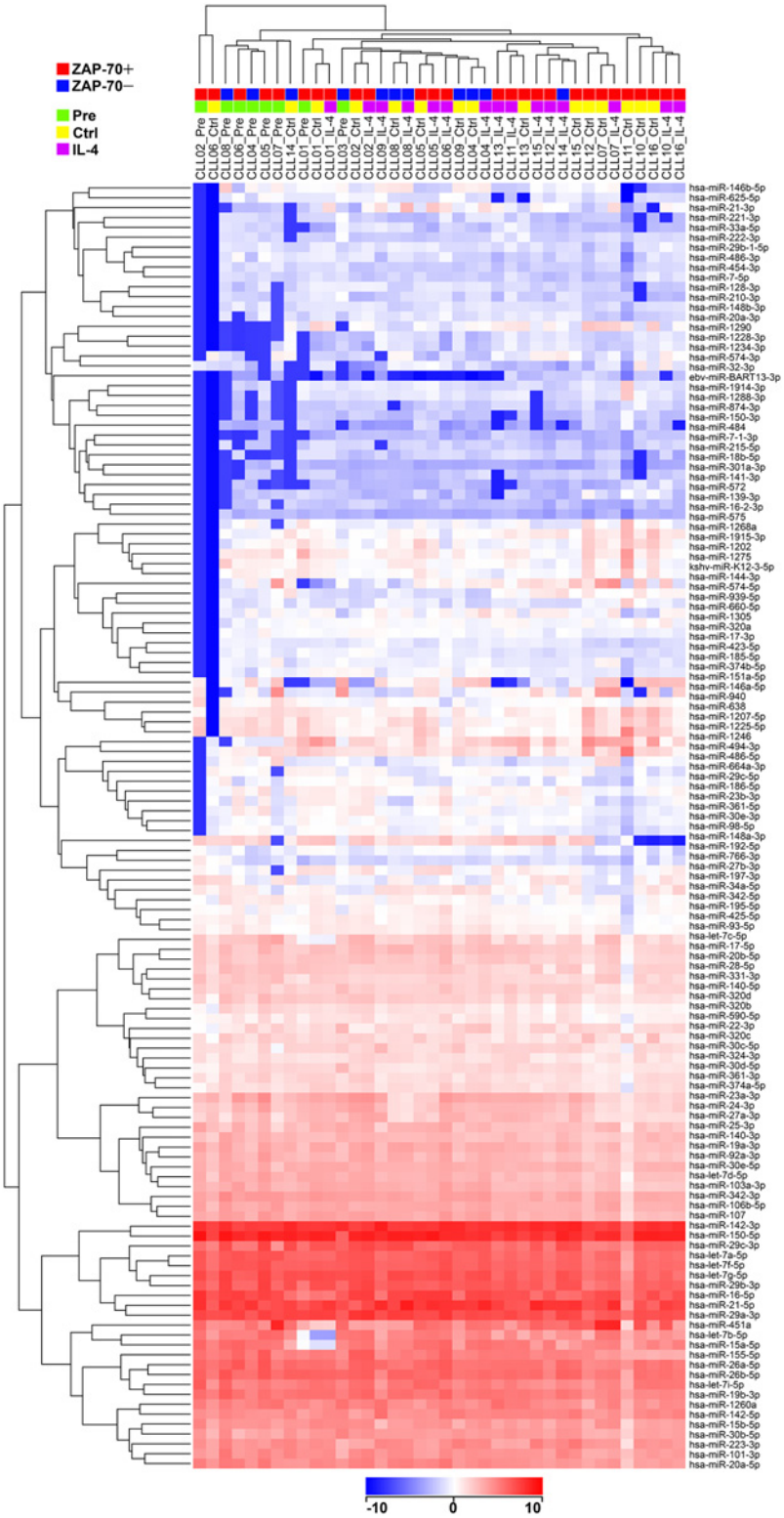

Fig 1. Hierarchical clustering analysis on the 129 miRNAs expressed in CLL using all the CLL samples. Microarray analysis was performed in 16 CLL patients, using different conditions: Pre (n = 8), Ctrl (n = 15), and IL-4 (n = 15), being all the Ctrl and IL-4 samples paired. The ZAP-70 status of the patients, positive (n = 11) and negative (n = 5), and the conditions of the samples, are indicated on the top. The relative levels of expression of miRNAs are depicted according to the shown $\log_2$ color scale on the bottom.

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Table 2. MiRNAs diferentially expressed in ZAP-70 positive and negative patients.

Mature miRNA ID	ZAP-70 Pos*	ZAP-70 Neg*	Fold change†	p value§
hsa-miR-29c-3p	53.79	93.01	-1.73	4.7E-02
hsa-miR-30b-5p	11.98	17.93	-1.50	2.0E-02
hsa-miR-320b	1.740	2.418	-1.39	4.6E-02
hsa-miR-29c-5p	0.473	1.102	-2.33	8.3E-03
hsa-miR-146b-5p	0.184	1.464	-7.96	1.3E-02
hsa-miR-210-3p	0.144	0.432	-3.01	4.7E-02

* Relative mean values of 11 ZAP-70 Pos and 5 ZAP-70 Neg cases, whose values were calculated as the mean of all available samples (Pre, Ctrl, IL-4).

†ZAP-70 Pos vs ZAP-70 Neg.

§Student t test ZAP-70 Pos vs ZAP-70 Neg.

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ordered according to their levels of expression (Fig 1). The 5p and 3p variants of a miRNA were often expressed at divergent levels and grouped in different sets, as is the case for miR-150-5p and miR-150-3p, or miR-21-5p and miR-21-3p. From the miR-17~92 cluster, miR-17-5p, miR-19a-3p, miR-19b-3p, miR-20a-5p, and miR-92a-3p were expressed at high levels, and miR-17-3p, and miR-20a-3p, at low levels. However, it was not a rule, since there were examples of paired 5p and 3p miRNAs expressed at similar levels, such as miR-142-3p and miR-142-5p, or miR-140-3p and miR-140-5p.

Correlations between miRNAs and spontaneous apoptosis induced by cell culture

The levels of spontaneous apoptosis (Ctrl samples) and following IL-4 treatment were measured in 13 out of the 16 patients studied by microarray. The percentages of apoptotic cells were 28.9±4.01 (mean±s.e.m.) for Ctrl samples and 8.87±1.04 for IL-4 samples. The relationship between spontaneous apoptosis and baseline miRNA expression was examined by Pearson correlation analysis, resulting in significant negative correlations for 29 miRNAs, most of them expressed at high levels, including miR-29a-3p, let-7g-5p, miR-29b-3p, let-7f-5p, let-7a-5p, miR-26b-5p, miR-19b-3p, or miR-155-5p, and positive correlations for 9 miRNAs, all of them expressed at low levels, including miR-1246, or miR-638 (S4 Table). These results suggest that both groups may play anti- or pro-apoptotic roles, respectively. The ANOVA test with post hoc analysis for condition Pre vs conditions Ctrl and IL-4 (cut-off 2-fold, $p<0.05$) using all the available microarray experiments from the 16 patients, identified 12 mature miRNAs up-regulated by cell culture in CLL (S5 Table). From this list, miR-1246 and miR-1290 were positively correlated with spontaneous apoptosis, further suggesting that they are candidates to play pro-apoptotic roles in CLL.

Identification of miRNA changes induced by IL-4 in CLL

The ANOVA test with post hoc analysis for condition IL-4 vs conditions Pre and Ctrl identified 7 mature miRNAs regulated by IL-4 in CLL (Fig 2A and S6 Table): miR-21-3p, miR-362-3p, miR-362-5p, miR-500a-3p, miR-502-3p, miR-532-3p, and miR-532-5p, all of them higher than 10-fold up-regulated on average. An identical set of 7 miRNAs had emerged in our first analysis, restricted to the 7 patients for which paired samples Pre, Ctrl and IL-4 were available (data not shown). Student paired t test for comparison IL-4 vs Ctrl (n = 15) identified an identical set of miRNAs and added to the list miR-21-5p (2.05-fold up-regulated), and miR-630 (9.95-fold down-regulated) (S6 Table). The latter was 94.6-fold higher for comparison Ctrl vs

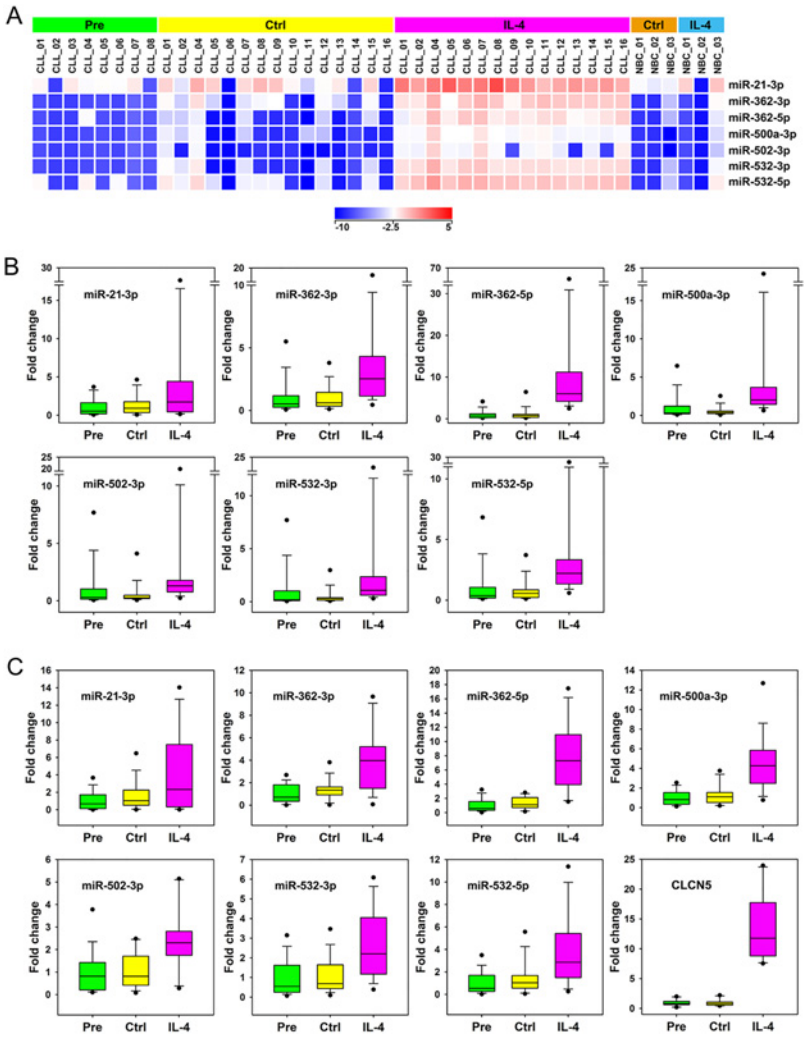

Fig 2. Identification of the miRNAs regulated by IL-4 in CLL. (A) Heat maps for expression of miRNAs significantly regulated by IL-4 in CLL (n = 15) following one-way ANOVA with Tukey HSD post hoc analysis for comparison of condition IL-4 vs Pre and Ctrl and above 2-fold change for both comparisons ($p<0.05$). Three NBC samples were included as controls. Relative expression levels are depicted according to the shown $\log_2$ color scale. (B, C) Validation of miRNAs regulated by IL-4 in CLL by qPCR, using RNU6-6P (B) and GAPDH (C) as references. Box whiskers representations of qPCR validations of the set of 7 miRNAs regulated by IL-4 in CLL are shown. Validation of up-regulation of the carrier gene CLCN5 by IL-4 in CLL, using GAPDH as reference, is included in C (right bottom panel). QPCR data are expressed as fold changes. For each assay, the average of Pre samples was set as 1. MiRNAs are ordered alphabetically.

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Pre, suggesting that IL-4, rather than inducing miR-630, partially counteracted its spontaneously induced up-regulation during cell culture. MiR-21 maps several hundred base pairs downstream of the last exon of VMP1, and miR-362, miR-500a, miR-502, and miR-532 within the third intron of CLCN5. Both are genes regulated by IL-4 according to our previous study [12], suggesting that regulation by IL-4 of these miRNAs is linked to that of their carrier genes. In general, their levels of change significantly correlated with each other (Table 3). In NBC, the whole set was up-regulated by IL-4 in 1 out of 3 cases (NBC_03) and miR-21-3p in another case (NBC_01) (Fig 2A).

Table 3. Correlations between the levels of cytoprotection and the amount of change in the levels of miRNAs by IL-4, and between the changes in the miRNAs with each other.

	Cytoprotection*	miR21-5p	miR21-3p	mi362-3p	mi362-5p	mi500a-3p	mi502-3p	mi532-3p	
R coefficient[§]	0.576								miR21-5p
p value	**3.95E-02**								
R coefficient	0.453	0.488							miR21-3p
p value	1.20E-01	9.07E-02							
R coefficient	0.311	0.655	0.679						mi362-3p
p value	3.02E-01	**1.51E-02**	**1.06E-02**						
R coefficient	0.559	0.621	0.528	0.625					mi362-5p
p value	**4.73E-02**	**2.35E-02**	6.36E-02	**2.24E-02**					
R coefficient	0.58	0.246	0.449	0.359	0.806				mi500a-3p
p value	**3.76E-02**	4.18E-01	1.23E-01	2.28E-01	**8.87E-04**				
R coefficient	0.444	0.412	-0.224	0.164	0.282	0.224			mi502-3p
p value	1.28E-01	1.62E-01	4.62E-01	5.93E-01	3.50E-01	4.62E-01			
R coefficient	0.548	0.585	0.542	0.639	0.996	0.834	0.283		mi532-3p
p value	5.26E-02	**3.58E-02**	5.55E-02	**1.86E-02**	**6.33E-13**	**3.95E-04**	3.48E-01		
R coefficient	0.493	0.636	0.654	0.918	0.829	0.619	0.223	0.841	mi532-5p
p value	8.68E-02	**1.95E-02**	**1.53E-02**	**9.41E-06**	**4.54E-04**	**2.41E-02**	4.65E-01	**3.16E-04**	

*Cytoprotection was calculated as the difference in apoptosis between conditions IL-4 vs Ctrl (n = 13)

[§]Pearson correlation analysis using fold changes between conditions IL-4 vs Ctrl expressed as $\log_2$ values; p values of less than 0.05 are typed in bold characters

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Validation of miRNAs and carrier genes regulated by IL-4 identified by microarray analysis

The miRNAs regulated by IL-4 were assayed by qPCR, using RNU6-6P (Fig 2B) and GAPDH (Fig 2C) as references. ANOVA analysis for comparison of condition IL-4 vs conditions Pre and Ctrl confirmed the significant up-regulation of all the miRNAs included in this set ($p<0.05$, data not shown). The commercial qPCR assays used in this study confirmed up-regulation of CLCN5 by IL-4 in CLL (Fig 2C, right bottom panel), though not that of VMP1 (data not shown). Validation of VMP1 up-regulation by IL-4 in CLL was achieved by means of a semiquantitative RT-PCR assay (Fig 3).

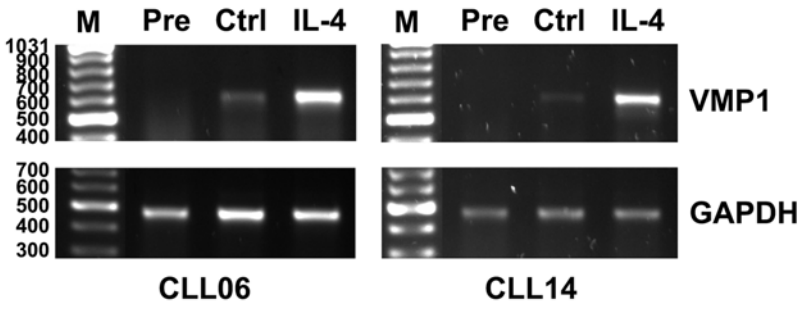


Fig 3. Validation of up-regulation of the carrier gene VMP1 by IL-4 in CLL. Two of the CLL patients, CLL06 and CLL14, were assayed by a semiquantitative RT-PCR assay. GAPDH was used as reference. The size of the molecular weight markers (M) is indicated to the left. The size of the expected PCR fragments were 606 bp for VMP1 and 464 bp for GAPDH.

doi:10.1371/journal.pone.0124936.g003

Correlations between ZAP-70 status, miRNA regulation by IL-4, and apoptosis

Correlation analyses between the levels of change of each miRNA and the baseline levels of expression of ZAP-70, and CD38, were performed to determine whether the miRNA response to IL-4 was related to CLL prognostic markers, but no significant correlations were found (data not shown). In contrast, the levels of cytoprotection correlated significantly with the levels of change of miR-21-5p, miR-362-5p, and miR-500a-3p (Table 3), providing additional evidence to suggest that these miRNAs could play a role in the anti-apoptotic response induced by IL-4 in CLL.

Discussion

IL-4 is a key survival signal provided to CLL cells by the microenvironment. IL-4 induces profound gene and protein expression changes, which confer cytoprotection against cell death induced spontaneously or by cytotoxic drugs. Here, we have studied how IL-4 affects miRNA expression, an essential regulatory component of gene and protein expression regulation, which may help to understand the mechanisms sustaining CLL pathogenesis. By the use of a microarray platform which allows measuring the levels of expression of 955 mature miRNAs, we first identified 129 miRNAs expressed at significant levels in CLL. The miRNAs expressed at the highest levels coincided with those reported by previous studies, and were similar to those expressed by NBC in our study, though several divergences emerged between CLL and NBC, such as the previously reported overexpression of miR-150-5p, miR-29a-3p, miR-155-5p, or miR-101-3p, underexpression of miR-181a-5p, or miR-181b-5p [14–19], and others not firmly established yet, including the highly divergent miR-451a, miR-28-5p, miR-144-5p, miR-486-5p, or miR-486-3p, within the overexpressed, and miR-126-3p, miR-365a-3p, miR-199a-3p, or miR-582-5p, within the underexpressed.

Hierarchical clustering analysis grouped the Pre samples together, and Ctrl and IL-4 samples mixed, illustrating that cell culture on its own induces changes, as previously observed for gene expression [12]. Mixed Ctrl and IL-4 samples moderately grouped according to ZAP-70 status, indicating that this marker has a broad impact on miRNA expression in CLL. This is substantiated by differential expression of several miRNAs according to ZAP-70 status, including both the 3p and 5p variants of miR-29c, which is recurrently found down-regulated in ZAP-70 positive patients, miR-146b-5p, as in the recent study by Negrini et al [25], and miR-210-3p. These results support their potential use as prognostic biomarkers.

Correlation analysis between the baseline levels of miRNAs and spontaneous apoptosis indicated a relatively high number of candidate miRNAs with a potential anti-apoptotic or pro-apoptotic function. From this list, most miRNAs need functional validation, using a similar approach as that provided for the cytoprotective effects of miR-17-5p in the study by Bomben et al [35]. IL-4 is a strong anti-apoptotic stimulus for CLL cells, and the responsible mechanism has not been elucidated yet, though gene expression changes are likely involved. Many genes regulated by IL-4 have been proposed as potentially involved [12], though most of them will also require direct assessment. Because miRNAs control gene expression, it is possible that miRNAs regulated by IL-4 contribute to gene expression changes and to the anti-apoptotic function. We have found up-regulation of miR-21 (5p and 3p), miR-362 (3p and 5p), miR-500a-3p, miR-502-3p, and miR-532 (3p and 5p) by IL-4 in CLL. All these miRNAs were also up-regulated in at least 1 out of 3 NBC, indicating that their regulation by IL-4 is not restricted to CLL cells.

MiR-21 was previously found overexpressed in the allergic lungs of diverse asthma mice models (transgenic IL-4 and IL-13 mice, wild-type mice treated with allergen, IL-4 or IL-13) [36], and was induced by IL-4 in B cells [37]. MiR-21 is produced from two types of primary

miR-21 transcripts, a pri-miR-21 arising from the miR-21 promoter located at the last introns of the VMP1 gene, and a VMP1-miR-21 transcript arising from the VMP1 promoter, both of which bypass the polyadenylation signals of VMP1 [38]. Because expression of VMP1 is induced by IL-4 [12], miR-21 up-regulation by IL-4 is likely produced through VMP1-miR-21 transcription. Concomitant up-regulation by IL-4 of the miRNAs comprised at the third exon of the CLCN5 gene suggests that they were likely produced also, rather than through transcription from their own promoters, as a consequence of being embedded within the CLCN5 primary transcript, another gene previously found up-regulated by IL-4 in CLL and NBC [12]. Thereafter, these primary transcripts would be successfully processed to produce the mature miRNAs.

MiR-21 is the miRNA most recurrently found overexpressed in human cancer [39], and its oncogenic role has been proven [27]. It has been found overexpressed in CLL [14,18], in association with promoter hypomethylation [40]. High level of expression of miR-21 associates to worse prognosis and chemotherapy resistance in CLL [41,42] and in cancer in general [43]. At the cellular level, an anti-apoptotic role has been proposed for miR-21, through repression of diverse pro-apoptotic targets, including PDCD4 and PTEN, resulting in activation of anti-apoptotic pathways such as Ras and NFκB [44]. Our data showed that miR-21-5p and some miRNAs hosted in the CLCN5 gene were significantly correlated to cytoprotection by IL-4 in CLL, further indicating these miRNAs as candidates to prevent cell death. This hypothesis should be directly assessed in future studies. Some of the miRNAs hosted in the CLCN5 gene have also been related to cancer, e.g., high expression of miR-362 is associated to worse prognosis and apoptosis resistance in colorectal and gastric cancer [45,46], that of miR-500a to hepatocellular carcinoma [47], and that of miR-502 to diverse types of cancer through a polymorphism in its target gene SET8 [48], and to colon cancer by inhibiting autophagy [49].

In summary, the present study identifies the miRNAs regulated by IL-4 in CLL and the probable responsible mechanism, contributing to the understanding of the anti-apoptotic response to IL-4, which could be relevant in evasion of apoptosis of CLL cells, resistance to chemotherapy, and leukemogenesis. Our data indicate that the IL-4 pathway and the miRNAs induced by IL-4 are promising targets for the development of novel therapies in CLL.

Supporting Information

S1 Fig. Cumulative distributions of mature miRNAs according to their signal intensity values. Microarrays performed on CLL and NBC were analysed with the flag "detected" set at "higher than 75%". The absence of miRNAs significantly expressed between intensity levels of −7 and −4 led us to exclude values inferior to −7 as not distinguishable from background, and to choose −4 as a positivity cut-off, which defined 129 and 149 miRNAs as significantly expressed in CLL and NBC.
(TIF)

S1 Table. Characteristics of CLL patients.
(XLSX)

S2 Table. MiRNAs expressed in CLL patients.
(XLSX)

S3 Table. MiRNAs expressed in NBC.
(XLSX)

S4 Table. Correlations between baseline expression of miRNAs and the levels of spontaneous apoptosis in CLL.
(XLSX)

S5 Table. MiRNA changes induced by cell culture not counteracted by IL-4 in CLL.
(XLSX)

S6 Table. MiRNA changes induced by IL-4 in CLL, and comparison with changes in NBC.
(XLSX)

Author Contributions

Conceived and designed the experiments: NRL MJAG AP. Performed the experiments: NRL MJAG SSR AMGS AM AP. Analyzed the data: NRL MJAG AP. Contributed reagents/materials/analysis tools: JGE JMM AMGA. Wrote the paper: AP.

References

1. Okada H, Banchereau J, Lotze MT. Interleukin-4. In Thompson AW, Lotze MT, editors. The Cytokine Handbook, Vol. I. London: Academic Press; 2003. pp 227–262.
2. Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G, Sarfati M. Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. J Exp Med. 1992; 176: 1319–1326. PMID: 1402678
3. Douglas RS, Capocasale RJ, Lamb RJ, Nowell PC, Moore JS. Chronic lymphocytic leukemia B cells are resistant to the apoptotic effects of Transforming Growth Factor-β. Blood. 1997; 89: 941–947. PMID: 9028325
4. Coscia M, Pantaleoni F, Riganti C, Vitale C, Rigoni M, Peola S, et al. IGHV unmutated CLL B cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. Leukemia. 2011; 25: 828–837. doi: 10.1038/leu.2011.12 PMID: 21372840
5. Steele AJ, Prentice AG, Cwynarski K, Hoffbrand AV, Hart SM, Lowdell MW, et al. The JAK3-selective inhibitor PF-956980 reverses the resistance to cytotoxic agents induced by interleukin-4 treatment of chronic lymphocytic leukemia cells: potential for reversal of cytoprotection by the microenvironment. Blood. 2010; 116: 4569–4577. doi: 10.1182/blood-2009-09-245811 PMID: 20716767
6. Chiorazzi N. Implications of new prognostic markers in chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program. 2012; 2012: 76–87. doi: 10.1182/asheducation-2012.1.76 PMID: 23233564
7. Zamorano J, Mora AL, Boothby M, Keegan AD. NF-kappa B activation plays an important role in the IL-4-induced protection from apoptosis. Int Immunol. 2001; 13: 1479–1487. PMID: 11717189
8. Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol. 2000; 164: 2200–2206. PMID: 10657675
9. Cuní S, Pérez-Aciego P, Pérez-Chacón G, Vargas JA, Sánchez A, Martín-Saavedra FM, et al. A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. Leukemia. 2004; 18: 1391–1400. PMID: 15175625
10. Thieu VT, Nguyen ET, McCarthy BP, Bruns HA, Kapur R, Chang CH, et al. IL-4-stimulated NF-kappaB activity is required for Stat6 DNA binding. J Leukoc Biol. 2007; 82: 370–379. PMID: 17513694
11. Bhattacharya N, Reichenzeller M, Caudron-Herger M, Haebe S, Brady N, Diener S, et al. Loss of cooperativity of secreted CD40L and increased dose-response to IL4 on CLL cell viability correlates with enhanced activation of NF-kB and STAT6. Int J Cancer. 2015; 136: 65–73. doi: 10.1002/ijc.28974 PMID: 24828787
12. Ruiz-Lafuente N, Alcaraz-García MJ, Sebastián-Ruiz S, Gómez-Espuch J, Funes C, Moraleda JM, et al. The gene expression response of chronic lymphocytic leukemia cells to IL-4 is specific, depends on ZAP-70 status and is differentially affected by an NFκB inhibitor. PLoS ONE. 2014; 9: e109533. doi: 10.1371/journal.pone.0109533 PMID: 25280001
13. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol. 2014; 9: 287–314. doi: 10.1146/annurev-pathol-012513-104715 PMID: 24079833
14. Fulci V, Chiaretti S, Goldoni M, Azzalin G, Carucci N, Tavolaro S, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood. 2007; 109: 4944–4451. PMID: 17327404
15. Marton S, Garcia MR, Robello C, Persson H, Trajtenberg F, Pritsch O, et al. Small RNAs analysis in CLL reveals a deregulation of miRNA expression and novel miRNA candidates of putative relevance in CLL pathogenesis. Leukemia. 2008; 22: 330–338. PMID: 17989717

16. Lawrie CH, Ballabio E, Dyar OJ, Jones M, Ventura R, Chi J, et al. MicroRNA expression in chronic lymphocytic leukaemia. Br J Haematol. 2009; 147: 398–402. doi: 10.1111/j.1365-2141.2009.07857.x PMID: 19681887

17. Vargova K, Curik N, Burda P, Basova P, Kulvait V, Pospisil V, et al. MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia. Blood. 2011; 117: 3816–3825. doi: 10.1182/blood-2010-05-285064 PMID: 21296997

18. Zhu DX, Zhu W, Fang C, Fan L, Zou ZJ, Wang YH, et al. MiR-181a/b significantly enhances drug sensitivity in chronic lymphocytic leukemia cells via targeting multiple anti-apoptosis genes. Carcinogenesis. 2012; 33: 1294–1301. doi: 10.1093/carcin/bgs179 PMID: 22610076

19. Li S, Moffett HF, Lu J, Werner L, Zhang H, Ritz J, et al. MicroRNA expression profiling identifies activated B cell status in chronic lymphocytic leukemia cells. PLoS ONE. 2011; 6: e16956. doi: 10.1371/journal.pone.0016956 PMID: 21408091

20. Smonskey MT, Block AW, Deeb G, Chanan-Khan AA, Bernstein ZP, Miller KC, et al. Monoallelic and biallelic deletions of 13q14.3 in chronic lymphocytic leukemia: FISH vs miRNA RT-qPCR detection. Am J Clin Pathol. 2012; 137: 641–646. doi: 10.1309/AJCPP31FSSRQTTAQ PMID: 22431542

21. Rodríguez AE, Hernández JÁ, Benito R, Gutiérrez NC, García JL, Hernández-Sánchez M, et al. Molecular characterization of chronic lymphocytic leukemia patients with a high number of losses in 13q14. PLoS ONE. 2012; 7: e48485. doi: 10.1371/journal.pone.0048485 PMID: 23152777

22. Dijkstra MK, van Lom K, Tielemans D, Elstrodt F, Langerak AW, van 't Veer MB, et al. 17p13/TP53 deletion in B-CLL patients is associated with microRNA-34a downregulation. Leukemia. 2009; 23: 625–627. doi: 10.1038/leu.2008.264 PMID: 18818704

23. Zenz T, Mohr J, Eldering E, Kater AP, Bühler A, Kienle D, et al. MiR-34a as part of the resistance network in chronic lymphocytic leukemia. Blood. 2009; 113: 3801–3808. doi: 10.1182/blood-2008-08-172254 PMID: 18941118

24. Mraz M, Malinova K, Kotaskova J, Pavlova S, Tichy B, Malcikova J, et al. MiR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. Leukemia. 2009; 23: 1159–1163. doi: 10.1038/leu.2008.377 PMID: 19158830

25. Negrini M, Cutrona G, Bassi C, Fabris S, Zagatti B, Colombo M, et al. MicroRNAome expression in chronic lymphocytic leukemia: comparison with normal B cell subsets and correlations with prognostic and clinical parameters. Clin Cancer Res. 2014; 20: 4141–4153. doi: 10.1158/1078-0432.CCR-13-2497 PMID: 24916701

26. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci USA. 2006; 103: 7024–7029. PMID: 16641092

27. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature. 2010: 467: 86–90. doi: 10.1038/nature09284 PMID: 20693987

28. Santanam U, Zanesi N, Efanov A, Costinean S, Palamarchuk A, Hagan JP, et al. Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. Proc Natl Acad Sci USA. 2010; 107: 12210–12215. doi: 10.1073/pnas.1007186107 PMID: 20566844

29. Sandhu SK, Fassan M, Volinia S, Lovat F, Balatti V, Pekarsky Y, et al. B-cell malignancies in microRNA Eμ-miR-17~92 transgenic mice. Proc Natl Acad Sci USA. 2013; 110: 18208–18213. doi: 10.1073/pnas.1315365110 PMID: 24145403

30. Raveche ES, Salerno E, Scaglione BJ, Manohar V, Abbasi F, Lin YC, et al. Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. Blood. 2007; 109: 5079–5086. PMID: 17351108

31. Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell. 2010; 17: 28–40. doi: 10.1016/j.ccr.2009.11.019 PMID: 20060366

32. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 2005; 353: 1793–1801. PMID: 16251535

33. Papakonstantinou N, Ntoufa S, Chartomatsidou E, Papadopoulos G, Hatzigeorgiou A, Anagnostopoulos A, et al. Differential microRNA profiles and their functional implications in different immunogenetic subsets of chronic lymphocytic leukemia. Mol Med. 2013; 19: 115–123. doi: 10.2119/molmed.2013.00005 PMID: 23615967

34. Stamatopoulos B, Meuleman N, De Bruyn C, Pieters K, Anthoine G, Mineur P, et al. A molecular score by quantitative PCR as a new prognostic tool at diagnosis for chronic lymphocytic leukemia patients. PLoS ONE. 2010; 5: e12780. doi: 10.1371/journal.pone.0012780 PMID: 20862275

35. Bomben R, Gobessi S, Dal Bo M, Volinia S, Marconi D, Tissino E, et al. The miR-17 ~ 92 family regulates the response to Toll-like receptor 9 triggering of CLL cells with unmutated IGHV genes. Leukemia. 2012; 26: 1584–1593. doi: 10.1038/leu.2012.44 PMID: 22343732

36. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol. 2009; 182: 4994–5002. doi: 10.4049/jimmunol.0803560 PMID: 19342679

37. Thapa DR, Bhatia K, Bream JH, D'Souza G, Rinaldo CR, Wolinsky S, et al. B-cell activation induced microRNA-21 is elevated in circulating B cells preceding the diagnosis of AIDS-related non-Hodgkin lymphomas. AIDS. 2012; 26: 1177–1180. doi: 10.1097/QAD.0b013e3283543e0e PMID: 22487708

38. Ribas J, Ni X, Castanares M, Liu MM, Esopi D, Yegnasubramanian S, et al. A novel source for miR-21 expression through the alternative polyadenylation of VMP1 gene transcripts. Nucleic Acids Res. 2012; 40: 6821–6833. doi: 10.1093/nar/gks308 PMID: 22505577

39. Pan X, Wang ZX, Wang R. MicroRNA-21: a novel therapeutic target in human cancer. Cancer Biol Ther. 2010; 10: 1224–1232. PMID: 21139417

40. Baer C, Claus R, Frenzel LP, Zucknick M, Park YJ, Gu L, et al. Extensive promoter DNA hypermethylation and hypomethylation is associated with aberrant microRNA expression in chronic lymphocytic leukemia. Cancer Res. 2012; 72: 3775–3785. PMID: 22710432

41. Rossi S, Shimizu M, Barbarotto E, Nicoloso MS, Dimitri F, Sampath D, et al. MicroRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. Blood. 2010; 116: 945–952. doi: 10.1182/blood-2010-01-263889 PMID: 20393129

42. Ferracin M, Zagatti B, Rizzotto L, Cavazzini F, Veronese A, Ciccone M, et al. MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia. Mol Cancer. 2010; 9: 123. doi: 10.1186/1476-4598-9-123 PMID: 20504344

43. Wang W, Li J, Zhu W, Gao C, Jiang R, Li W, et al. MicroRNA-21 and the clinical outcomes of various carcinomas: a systematic review and meta-analysis. BMC Cancer. 2014; 14: 819. doi: 10.1186/1471-2407-14-819 PMID: 25376700

44. Buscaglia LE, Li Y. Apoptosis and the target genes of microRNA-21. Chin J Cancer. 2011; 30: 371–380. PMID: 21627859

45. Christensen LL, Tobiasen H, Holm A, Schepeler T, Ostenfeld MS, Thorsen K, et al. MiRNA-362-3p induces cell cycle arrest through targeting of E2F1, USF2 and PTPN1 and is associated with recurrence of colorectal cancer. Int J Cancer. 2013; 133: 67–78. doi: 10.1002/ijc.28010 PMID: 23280316

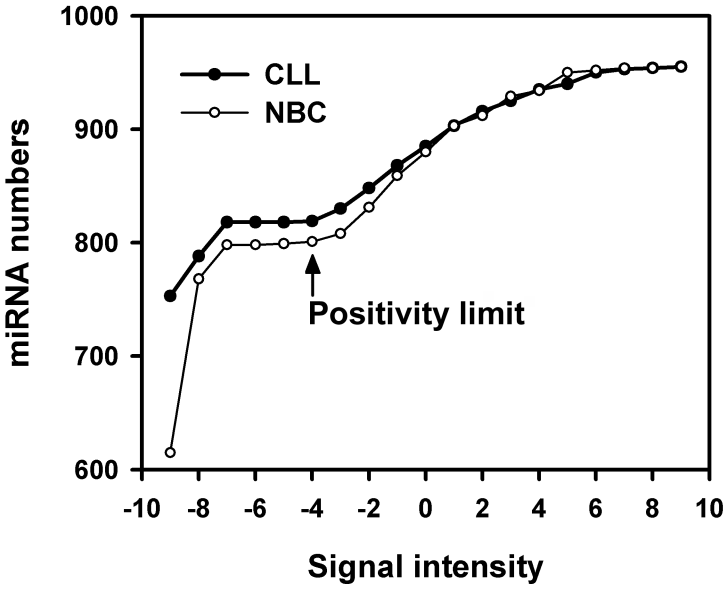
46. Xia JT, Chen LZ, Jian WH, Wang KB, Yang YZ, He WL, et al. MicroRNA-362 induces cell proliferation and apoptosis resistance in gastric cancer by activation of NF-κB signaling. J Transl Med. 2014; 12: 33. doi: 10.1186/1479-5876-12-33 PMID: 24495516

47. Yamamoto Y, Kosaka N, Tanaka M, Koizumi F, Kanai Y, Mizutani T, et al. MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma. Biomarkers. 2009; 14: 529–538. doi: 10.3109/13547500903150771 PMID: 19863192

48. Song F, Zheng H, Liu B, Wei S, Dai H, Zhang L, et al. An miR-502-binding site single-nucleotide polymorphism in the 3'-untranslated region of the SET8 gene is associated with early age of breast cancer onset. Clin Cancer Res. 2009; 15: 6292–6300. doi: 10.1158/1078-0432.CCR-09-0826 PMID: 19789321

49. Zhai H, Song B, Xu X, Zhu W, Ju J. Inhibition of autophagy and tumor growth in colon cancer by miR-502. Oncogene. 2013; 32: 1570–1579. doi: 10.1038/onc.2012.167 PMID: 22580605

Figure S1


1000
900
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miRNA numbers
CLL
NBC
Positivity limit
-10
-8
-6
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Signal intensity