UAGro
UNIVERSIDAD AUTÓNOMA DE GUERRERO

FACULTAD DE CIENCIAS QUÍMICO BIOLÓGICAS
DOCTORADO EN CIENCIAS BIOMÉDICAS

# Efecto de la sobre-expresión de DNMT3B en la actividad transcripcional de genes relacionados con el cáncer

# T E S I S

QUE PARA OBTENER EL GRADO DE

**DOCTORA EN CIENCIAS BIOMÉDICAS**


**P R E S E N T A**

***M en C. IRLANDA PERALTA ARRIETA***

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# UNIVERSIDAD AUTÓNOMA DE GUERRERO
## UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
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## DOCTORADO EN CIENCIAS BIOMÉDICAS

ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 20 días del mes de enero del dos mil dieciséis, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Efecto de la sobre-expresión de DNMT3B en la actividad transcripcional de genes relacionados con el cáncer"**, presentada por la alumna Irlanda Peralta Arrieta, para obtener el Grado de Doctora en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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# *Efecto de la sobre-expresión de DNMT3B en la actividad transcripcional de genes relacionados con el cáncer*

# ÍNDICE

## CONTENIDO

# RESUMEN

**Introducción:** DNMT3B, DNA metiltransferasa *de novo*, está frecuentemente sobre-expresada en cáncer. Dicho evento se asocia con metilación anormal de genes supresores de tumor y reparadores de DNA favoreciendo el desarrollo y progresión de varios tipos de cáncer humano.

**Objetivo:** Analizar el efecto de la sobre-expresión de DNMT3B en la expresión global de genes en células HaCaT y en la metilación de genes relacionados con el cáncer para la identificación de genes blanco de DNMT3B.

**Metodología**: La sobre-expresión de DNMT3B en la línea celular HaCaT se realizó con un vector de expresión, posteriormente se analizó el perfil global de expresión de genes mediante un microarreglo. Se identificaron los genes que disminuyeron su expresión debido a la sobre-expresión de DNMT3B y a partir de estos genes se hicieron análisis computacionales para identificar los procesos o vías celulares en los que participan, y además se identificó la presencia de isla CpG en el promotor de cada gen. En un grupo de 10 de genes se validaron los resultados del microarreglo por RT-qPCR y en 3 de éstos se hizo un análisis detallado de la metilación de su promotor por PCR-SM y modificación secuenciación. Finalmente se analizó la expresión de DNMT3B, VAV3, GPR137 y SORBS2 por RT-qPCR en líneas celulares de cáncer y en muestras de cáncer cervical.

**Resultados:** El análisis global de expresión de genes mostró que la sobre-expresión de DNMT3B en la línea celular HaCaT, resultó en la disminución de la expresión de 1085 genes. De éstos, se identificaron 151 genes con isla CpG en su promotor. Estos genes participan en procesos de comunicación celular, procesos celulares y procesos metabólicos que se ven afectados en el desarrollo de cáncer. Los datos de validación por RT-qPCR mostraron que los genes VAV3, GPR137 y SORBS2 fueron regulados negativamente por DNMT3B. Se encontró aumento en la metilación del promotor de VAV3, en12 sitios CpG cercanos al sitio de inicio de la transcripción después de la sobre-expresión de DNMT3B, mientras que, no se encontró incremento de la metilación en el promotor de GPR137 y SORBS2.

Adicionalmente, reportamos que la expresión de DNMT3B está aumentada en cáncer cervical y varias líneas celulares de cáncer, mientras que, la expresión de VAV3, GPR137 y SORBS2 está disminuida en líneas celulares de cáncer.

**Conclusiones:** La sobre-expresión de DNMT3B en células HaCaT afecta la expresión de genes con funciones que se ven afectadas en cáncer, como son: comunicación celular, procesos celulares y procesos metabólicos. DNMT3B disminuyó la expresión de 151 genes con isla CpG, y disminuyó la expresión del gen VAV3 vía metilación de su promotor, por lo que VAV3 podría ser considerado un gen blanco de DNMT3B. Los datos reportados en este trabajo, sugieren la importancia de DNMT3B en la regulación de la expresión de genes y en cáncer.

# INTRODUCCIÓN

En los mamíferos, la metilación del DNA consiste en la adición de un grupo metilo (-$CH_3$) en el carbono 5´de las citosinas, principalmente en el contexto de dinucleótidos CpG, para formar 5-metilcitosina. Los dinucleótidos CpG se agrupan en regiones llamadas islas CpG (Bird, 1987), y se localizan generalmente en el extremo 5´de los promotores de genes (Larsen et al., 1992, Gardiner-Garden and Frommer, 1987).

Una isla CpG se puede definir como, una región de más de 200 pb con un contenido de G+C del 50% y una relación esperada$_{CpG}$/observada$_{CpG}$ del 60% (Gardiner-Garden and Frommer, 1987). Sin embargo, el estudio de Takai y Jones (2002), propone que una isla CpG, debe ser considerada como una región de más de 500 pb con un contenido de G+C del 55% y una relación esperada$_{CpG}$/observada$_{CpG}$ del 65% Este criterio excluye a las secuencias *Alu* que pueden representar falsas islas CpG, y además, reduce el número de promotores de genes asociados a isla CpG (figura 1) (Takai and Jones, 2002).

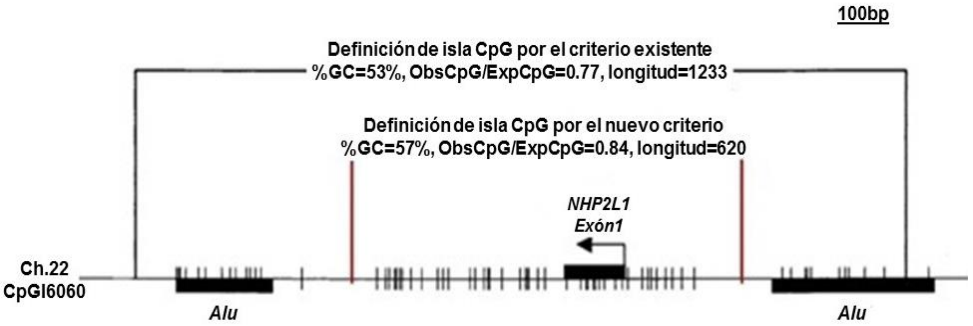


**Figura 1. Propuesta del criterio de isla CpG según Takai y Jones.** Una isla CpG puede considerarse como una región mayor o igual a 500 pb con un contenido de G+C mayor o igual al 55% y una relación esperada$_{CpG}$/observada$_{CpG}$ del 65% y que se asocia con la región 5´de genes. Excluye las secuencias *Alu* previamente identificadas en el extremo 5´de las islas CpG. En la figura se muestra como ejemplo un fragmento de DNA de 1233 pb del gen NHP2L1, calculado por el algoritmo, se descartan dos secuencias *Alu* con algunos CpGs. Este criterio de isla reduce el tamaño a 620 pb para este gen. Modificado de (Takai and Jones, 2002).

Se ha considerado a las islas CpG como una región reguladora de la transcripción, debido a que su alto contenido de CG, permite la posibilidad de presentar múltiples sitios de unión para factores de la transcripción ubicuos con un sitio CpG en su secuencia de unión a DNA, ejemplo de éstos son: Sp1, NRF-1, E2F, ETS (transcription factor-binding motifs), BoxA, CRE y E-box. Además, las

islas CpG muestran propiedades de cromatina abierta o permisiva para la transcripción (Deaton and Bird, 2011).

Aproximadamente, en el 60% de los promotores de genes humanos, hay al menos una isla CpG, entre los cuales se encuentran genes constitutivos, oncogenes y genes supresores de tumor (Antequera, 2003, Deaton and Bird, 2011). En una célula somática no tumoral, las islas CpG tienen como característica que no se encuentra metilada, o presentan un bajo contenido de CpGs metilados, permitiendo la transcripción del gen, sin embargo, en algunas patologías y procesos cancerosos ocurren cambios en los patrones de metilación del DNA, y las islas CpG llegan a ser metiladas de manera anormal inhibiendo la transcripción del gen (figura 2) (Deaton and Bird, 2011)

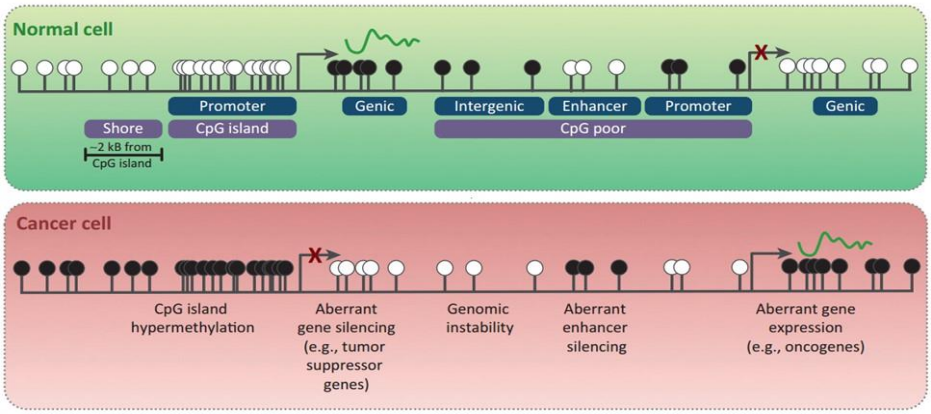


**Figura 2. Representación esquemática de los cambios en la metilación que ocurren en una célula cancerosa.** Las islas CpG están generalmente asociadas a promotores de genes y son resistentes a la metilación del DNA en una célula somática no tumoral (área sombreada en verde). En la región intergénica del gen, existe una región de menor densidad en CpG (CpG-poor), las cuales se encuentran típicamente metiladas en células normales para dar estabilidad genómica, es decir, de esta manera se evita que falsos sitios de la transcripción sean activados y se generen la expresión de oncogenes o secuencias de DNA repetitivo. En las células cancerosas, las islas CpG de genes supresores de tumor son propensas a una metilación anormal generando una estructura de cromatina cerrada, la cual resulta en el silenciamiento transcripcional del gen (área sombreada en rojo). Por otro lado, también ocurre hipometilación de las regiones intergénicas y de las áreas bajas en CpG que contribuye a la inestabilidad genómica y expresión aberrante de genes (por ejemplo, oncogenes). Los círculos blanco representan CpGs no metilado, los círculos negros CpGs metilados, tomado de (Stirzaker et al., 2014).

La metilación del DNA, es la principal modificación epigenética que en los mamíferos es esencial para el mantenimiento de la integridad del genoma (Kaneda et al., 2004), procesos del desarrollo (Chen et al., 2003, Li et al., 1992), y tiene un papel muy importante en la regulación de la expresión de genes, principalmente asociado al silenciamiento transcripcional (Herman et al., 1995, Yoshiura et al.,

1995), relacionado con el inicio y progresión de algunas patologías como el cáncer (Jones and Baylin, 2007).

De manera general, la metilación anormal en las islas CpG inhibe la transcripción, debido a que la 5-metilcitosina de los dinucleótidos CpG se encuentra en el surco mayor de la hélice de DNA, y esto hace que esta modificación directamente interfiere con la unión de las proteínas y los factores de la transcripción en el promotor del gen. Además, la metilación de las islas CpG provoca que la cromatina se compacte, generando un estado de cromatina silente y por tanto el silenciamiento transcripcional del gene (Bird, 2002, Hermann et al., 2004).

Otra manera en que la metilación del DNA afecta la expresión de genes, consiste en que la metilación que ocurre en el CG de la secuencia de unión para factores de la transcripción, puede interferir estéricamente con la unión de la proteína al DNA, disminuyendo o inhibiendo la transcripción del gen. En este sentido, se conocen algunos factores de la transcripción sensibles a metilación: AP-2, c-Myc/Myn, CREB/ATF, EBP-80, E2F, MIB-1, MLTF/USF, NF-κB, VBP1. Para otros factores de la transcripción, en cambio, la metilación en el CG de su secuencia de unión no interfiere con su unión al DNA. Ejemplo de estos factores son: CTF, Sp1 y TCR-ATF (Michael Holler et al., 1988, Tate and Bird, 1993, Zhu et al., 2003). Otro mecanismo de inhibición de la transcripción por metilación, involucra a las proteínas con dominio de unión a CpG metilado (MBD). Las proteínas MBD1-3 y MeCP2 se unen a los CpGs metilados y posteriormente reclutan diversos represores transcripcionales, como las deacetilasas de histonas (HDACs) y otros complejos remodeladores de la cromatina que mantienen una cromatina compacta impidiendo el acceso para las proteínas de inicio de la transcripción (Boyes and Bird, 1992, Rountree et al., 2001).

En comparación con una célula normal, las células cancerosas muestran cambios importantes en el estado de metilación del DNA, generalmente ocurre hipometilación global del genoma acompañado por la metilación regional en las islas CpG de promotores de genes supresores de tumor. La metilación anormal de genes involucrados en el control del ciclo celular, apoptosis, reparación del DNA, metástasis, resistencia a drogas, y otras vías involucradas en procesos tumorales

resulta en el silenciamiento transcripcional y en la pérdida de la función del gen, favoreciendo el inicio y progresión del cáncer (Cheung et al., 2009, Jones and Baylin, 2002). Este evento se ha observado virtualmente en cada tipo de tumor humano (tabla 1), y sus líneas celulares, y se ha considerado como una marca epigenética común en varios tipos de cáncer humano (Esteller, 2007, Esteller, 2011, Jones and Baylin, 2002). En base a lo anterior, diversos estudios han sugerido que, la metilación de genes muestra el potencial para proporcionar una nueva generación de biomarcadores, permitiendo la detección temprana de cáncer, determinar el pronóstico y la predicción de respuesta a terapia (Chen et al., 2014b, Dmitriev et al., 2015, Duffy et al., 2009, Huang et al., 2009, Oka et al., 2006, Wentzensen et al., 2009, Yanez et al., 2015).

**Tabla 1. Genes metilados en diferentes tipos de cáncer humano**

| Gen | Función | Tipo de cáncer | Gen | Función | Tipo de cáncer |
|---|---|---|---|---|---|
| APC | Señalización WNT | Próstata, colon, pulmón, vejiga | SNCG | Control del crecimiento celular | Mama, ovario |
| AR | Señalización receptor andrógeno | próstata | SOCS1 | Señalización citosina | Hígado |
| BMAL1 | Señalización AHR | Leucemia, linfoma | TFPI1 | Proteína de matriz extracelular | Colon |
| BRCA1 | Respuesta de daño a DNA | Mama, ovario | THBS1 | Proteína de matriz extracelular | Glioma |
| CDH1 | Adhesión célula-célula | Mama, próstata | TIG1 | Receptor de respuesta a ácido retinoico | Próstata |
| CDH11 | Adhesión célula-célula | Colon, mama, esófago, gástrico, hígado | TIMP2 | Inhibidor metalopeptidasa | Próstata |
| CDH13 | Adhesión célula-célula | Pulmón, cabeza y cuello | TP73 | Respuesta a estrés | Linfoma |
| CDKN2A | Control del ciclo celular | Linfoma, colon, estómago, próstata | TSHR | Receptor TSH | Tiroides |
| CDKN2B | Control del ciclo celular | Leucemia | VHL | Respuesta a hipoxia | Riñón |
| DAPK1 | Control de muerte celular programada | Pulmón, cabeza y cuello, vejiga | WIF1 | Señalización WNT | Colon |
| EMP3 | Transducción de señales | Glioma | WRN | DNA helicasa | Colon |
| ESR1 | Señalización del receptor de estrógenos | Mama | *P15*$^{NK4b}$ | Control del ciclo celular | Leucemia |
| GSTP1 | Detoxificación | Próstata, hígado, pulmón | PRLR | Receptor de prolactina | Mama |
| IGFBP3 | Transducción de señales | Colon, pulmón, ovario, próstata | Rb | Control del ciclo celular | Retinoblastoma |
| LGALS3 | Proteína de matriz extracelular | Próstata | FAT | Supresor de tumor, cadherina | Colon |
| MASPIN | Inhibidor peptidasa | Páncreas | DKK1 | Señalización WNT | Colon |
| MGMT | Reparación de DNA | Colon, glioma, linfoma, próstata, pulmón | COX2 | Ciclooxigenasa-2 | Colon, estómago |
| miR-148a | Supresor de metástasis | Metástasis | GATA4 y GATA5 | Factor de transcripción | Colon, estómago |
| miR-9 | Supresor de metástasis | Metástasis | ID4 | Factor de transcripción | Leucemia, estómago |
| miR-200s | Supresor de metástasis | Colon, vejiga, carcinoma de células escamosas | SRBC | Proteína de unión a BRCA1 | Mama, pulmón |
| MLH1 | Reparación de DNA | Colon, endometrio, estómago | SYK | Tirosina quinasa | Mama |
| NORE1A | Control de crecimiento celular | Colon, hígado, pulmón, tiroides | TMS1 | Apoptosis | Mama |
| NSD1 | Receptor nuclear | Glioma, neuroblastoma | SLC5A8 | Transportador de sodio | Glioma, colon |
| PYCARD | Apoptosis | Glioma, mama, colon, gástrico, pulmón | HOXA9 | Proteína homeobox | Neuroblastoma |
| RARB | Receptor de ácido retinoico | Mama, colon, próstata | EXT1 | Síntesis de heparán sulfato | Leucemia, piel |
| RASSF1A | Ciclo celular, reparación de DNA | Mama, ovario, pulmón, próstata, colon | Lamina A/C | Filamento intermedio nuclear | Linfoma, leucemia |
| RBP1 | Control de crecimiento celular | Linfoma, gástrico, carcinoma de células escamosas | RIZ1 | Metiltransferasa de histona | Leucemia, hígado, tiroides, gástrico, próstata |
| S100P | Control del ciclo celular | Páncreas | SEPT9 | Control del ciclo celular | Colon |

Recopilación de datos, modificado de (Esteller, 2007, Esteller, 2011, Heyn and Esteller, 2012).

Las DNA metiltransferasas (DNMTs) son las enzimas responsables de establecer y mantener los patrones de metilación en el genoma humano (Robertson, 2001). Tres DNMTs se han estudiado de manera significativa, tanto en el desarrollo normal de los mamíferos como su papel en cáncer y otras enfermedades (Chen et al., 2003, Vertino et al., 1996, Zhang et al., 2011). DNMT1 o DNMT de mantenimiento, muestra preferencia por el DNA hemimetilado y copia los patrones de metilación durante la replicación del DNA (Jurkowska et al., 2011). Las DNMTs *de novo*: DNMT3A y DNMT3B, tienen preferencia por los CpGs no metilados y establecen los patrones nuevos de metilación durante el desarrollo embrionario (Chen et al., 2003).

Las DNMTs están formadas por un dominio regulador (N-terminal) y un dominio catalítico (C-terminal). El dominio N-terminal difiere en tamaño entre las DNMTs y es el responsable de la interacción con el DNA y otras proteínas. El dominio regulador de DNMT1, DNMT3A y DNMT3B puede interactuar con proteínas remodeladoras de la cromatina y reguladores transcripcionales, mientras que DNMT1 a diferencia de las DNMTs *de novo*, interactúa, además con proteínas reguladoras del ciclo celular y con la maquinaria de replicación del DNA (Datta et al., 2005, Dhayalan et al., 2010, Fuks F et al., 2001, Fuks et al., 2003, Vertino et al., 2002). El dominio C-terminal implicado en la función catalítica está formado por una estructura común dependiente de actividad metiltransferasa (AdoMet dependent methyltransferase), que incluye seis dominios evolutivamente conservados entre las DNMTs: I, IV, VI, VIII, IX y X (figura 3). Estos subdominios catalizan la transferencia del grupo metilo donado por el cofactor S-adenosilmetionina (SAM) a las citosinas dentro de los dinucleótidos CpG (Hermann et al., 2004, Turek-Plewa and Jagodzinski, 2005).

Las DNMTs, muestran niveles bajos de expresión en células somáticas. Sin embargo, en células cancerosas y líneas celulares de cáncer, la expresión de las DNMTs, en especial de DNMT3B, está frecuentemente elevada. Esto se ha sugerido como un mecanismo potencial para el incremento de la metilación *de novo* de las islas CpG en los promotores de genes supresores de tumor en cáncer (Robertson et al., 1999, Subramaniam et al., 2014).

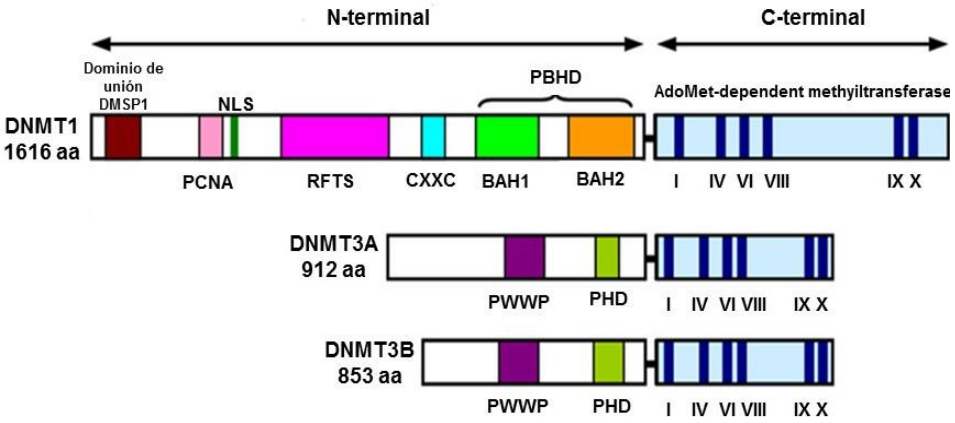


**Figura 3. Representación esquemática de la estructura de las DNA metiltransferasas de humano.** En el dominio N-terminal se muestran los subdominios de interacción con el DNA o proteínas. El dominio C-terminal contiene los dominios conservados entre las DNMTs. Modificado de (Ryazanova et al., 2013).

La sobre-expresión de DNMT3B se ha reportado en varios tipos de cáncer humano y sus líneas celulares como son: cáncer colorectal, mama, cervical, retinoblastoma, gástrico, oral, hepático, renal, cáncer de pulmón, melanoma y otros (el-Deiry et al., 1991, Micevic et al., 2016, Chen et al., 2014a, Gao et al., 2013, Girault et al., 2003, Oh et al., 2007, Qu et al., 2010, Robertson et al., 1999, Saito et al., 2002). DNMT3B, a diferencia de las otras DNMTs, específicamente contribuye con la carcinogénesis manteniendo el fenotipo transformante, y la supervivencia de las células cancerosas tanto *in vivo,* como *in vitro*, a través de la metilación *de novo* y el silenciamiento transcripcional de genes supresores de tumor (Beaulieu et al., 2002, Linhart et al., 2007, Nosho et al., 2009, Roll et al., 2008).

La relación de la expresión de DNMT3B con la metilación de genes en cáncer se ha demostrado (Ibrahim et al., 2011). La eliminación del gen de DNMT3B lleva a la demetilación y reactivación de genes supresores de tumor (Rhee et al., 2002, Xu et al., 2005, Garzon et al., 2009). En cáncer de esófago, la disminución de la expresión de los genes p14ARF y p16INK4a, implicados en el control del ciclo celular, se correlaciona con la sobre-expresión de DNMT3B (de Almeida Simão et al., 2006). En cáncer de colon, la expresión de DNMT3B se asocia con el fenotipo metilador (CpG island methylator phenotype, CIMP) (Nosho et al., 2009), mientras que en cáncer de mama, la sobre-expresión de la proteína de DNMT3B contribuye significativamente a una actividad elevada de DNMT y como consecuencia a la metilación anormal de varios genes (Roll et al., 2008). El papel de DNMT3B en la transformación celular también se ha demostrado. DNMT3B contribuye y acelera la

transformación celular inducida por carcinógenos del tabaco (Teneng et al., 2015), y por el antígeno SV40T en líneas celulares de cáncer de pulmón a través de la metilación de genes específicos (Soejima et al., 2003). De manera muy importante, el valor predictivo en el pronóstico que puede proporcionar DNMT3B a pacientes con un tipo de tumor también ha sido documentado. En este sentido, la elevada expresión de DNMT3B se asocia significativamente con una incidencia alta de metástasis de nódulos linfáticos, una recurrencia alta después del tratamiento y una corta supervivencia para pacientes con cáncer oral (Chen et al., 2014a). De la misma manera, la elevada expresión de DNMT3B, se asocia con menor supervivencia en pacientes con melanona (Micevic et al., 2016). Estas evidencias demuestran el importante papel que tiene DNMT3B en la metilación *de novo* de las islas CpG de genes supresores de tumor en diversos tipos de cáncer humano.

A pesar de la creciente lista de genes reportados como metilados en diferentes tipos de cáncer humano (tabla 1), y el importante papel de DNMT3B en cáncer, hasta ahora se han identificado 5 genes como blancos para la regulación transcripcional por DNMT3B, los cuales tienen un papel importante en la carcinogénesis: MTSS1 (Fan et al., 2012), HOXB13 (Ghoshal et al., 2010), Igf2 y Sfrp2 (Linhart et al., 2007), y MAL (Teneng et al., 2015). Otros genes asociados a cáncer, han sido reportados como silenciados transcripcionalmente vía metilación por DNMT3B, éstos son: RECK (Chang et al., 2006), MLH1 (Fang et al., 2014), y RASSF1A (Palakurthy et al., 2009).

Dado al importante papel de DNMT3B en la carcinogénesis, y debido a que todavía se desconocen muchos genes que pueden ser regulados transcripcionalmente de manera directa por DNMT3B, para encontrar genes blanco de esta enzima, en este trabajo se propuso que, la sobre-expresión de DNMT3B en la línea celular no tumoral HaCaT, resultaría en la metilación de genes relacionados con el cáncer, que tengan una isla CpG en su promotor, y sitios de unión para factores de la transcripción que pueden unirse a DNMT3B. La identificación de genes blanco de está DNA metiltransferasa *de novo*, resulta importante para entender tanto la función del gen, así como la participación de DNMT3B en las etapas de la carcinogénesis. Por lo que, el objetivo de este trabajo fue, valorar el efecto de la sobre-expresión de DNMT3B en células HaCaT en la expresión global

de genes y en la metilación para la identificación de genes relacionados con el cáncer como blancos de DNMT3B.

Para la selección de los posibles genes blanco de DNMT3B se siguió la logística que se muestra en el diagrama 1. Considerando los criterios de selección y exclusión que se muestran en el diagrama, seleccionamos finalmente tres genes para los análisis que permitieron identificar a los genes VAV3, GPR137 y SORBS2 como genes regulados negativamente y posibles blancos de DNMT3B en la línea celular HaCaT.

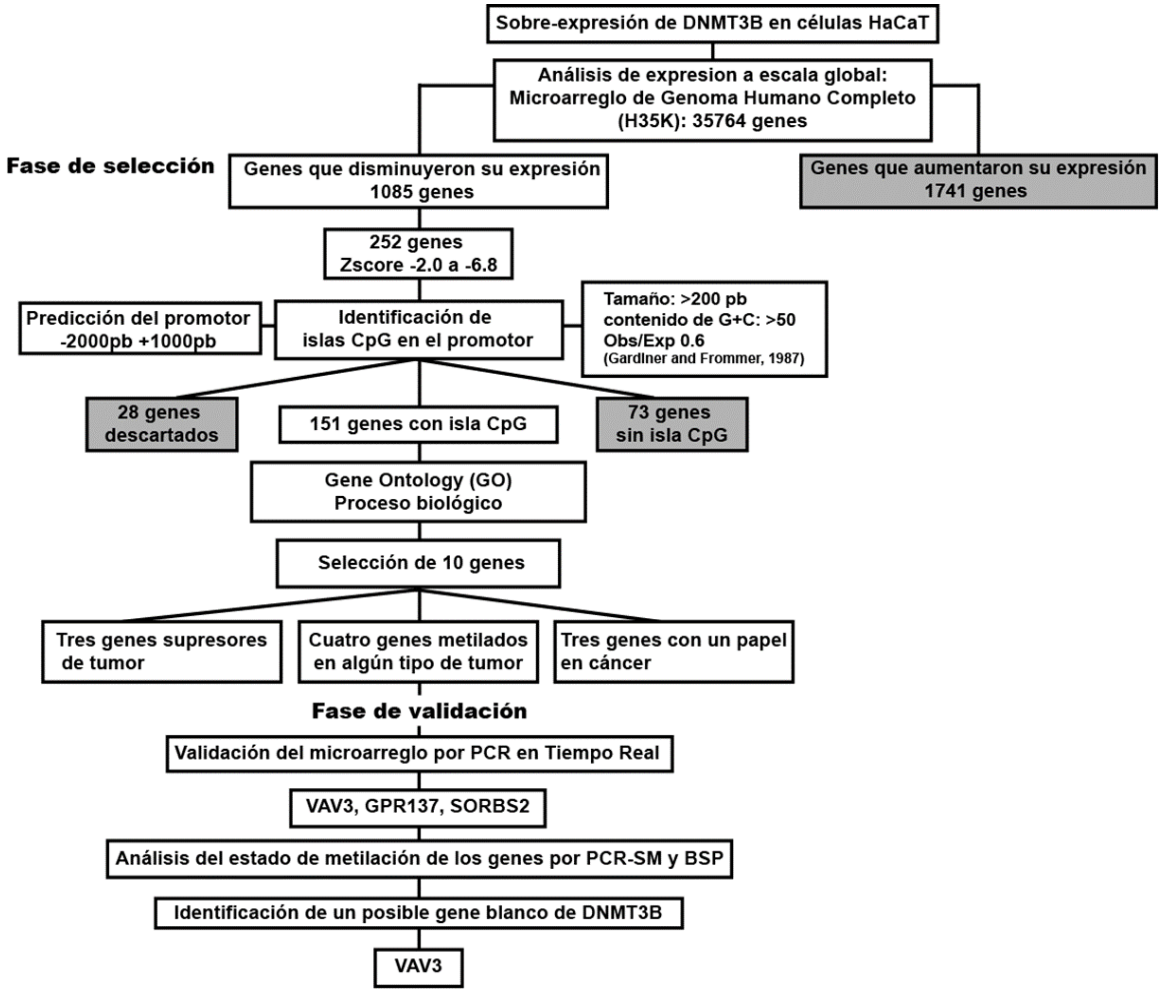


**Diagrama 1.** Se muestra un resumen de la logística que se utilizó en este trabajo para la selección de los posibles genes blanco de DNMT3B.Lo que se muestra en los cuadros en gris fueron criterios de exclusión, los cuadros en blanco se consideraron como los criterios de inclusión o selección.

A continuación se describe la participación en cáncer de los genes que fueron objeto de estudio en este trabajo.

**VAV3 (Guanine nucleotide exchange factor 3).** Es una proteína que en el humano es codificada por el gen VAV3, un integrante de la familia de genes VAV, ubicado en el cromosoma 1, en la posición p13.3 (1p13.3). VAV3 participa en vías activadas por proteínas tirosina quinasas (Movilla and Bustelo, 1999). La proteína VAV3, es un factor intercambiador de nucleótido de guanina (GEFs) para la familia de guanosina trifosfatasa (GTPasas) Rho y RhoA, las cuales son enzimas que tienen un papel en el rearreglo del citoesqueleto y en transcripción de genes (Denkinger et al., 2000, Movilla and Bustelo, 1999).

El papel de VAV3 en cáncer se ha reportado como un oncogén y está sobre-expresado (Uen et al., 2015). En cáncer de próstata activa el receptor andrógeno, y estimula el crecimiento celular (Dong et al., 2006). En cáncer de mama, activa al receptor de estrógenos a través de la vía PI3K-Akt, y también estimula el crecimiento celular (Lee et al., 2008).

Poco se sabe de la regulación de VAV3 en cáncer, sin embargo, existen algunos reportes donde han encontrado metilado el promotor del gen VAV3, y la disminución de su expresión en líneas celulares de cáncer de mama (Loss et al., 2010), y cáncer gástrico (Zong et al., 2016).

**SORBS2 (sorbin and SH3 domain containing 2)**. La proteína sorbina humana, es una forma alternativa de procesamiento de un transcrito del locus SORBS2/ArgBP2 ubicado dentro del cromosoma 4 en la región q35.1 (4q35.1). SORBS2, es una proteína que pertenece a la familia de proteínas adaptadoras SoHo, que incluye a dos integrantes más, Vinexina y CAP (c-Cbl associated protein)/Ponsina. Estas proteínas muestran la misma organización estructural con un dominio SoHo (Sorbin Homology) en su región N-terminal y tres dominios SH3 en su región C-terminal (Kioka et al., 2002). El dominio SH3 de SORBS2 es muy similar a los dominios encontrados en las proteínas que regulan el citoesqueleto de actina, además regulan la unión de SORBS2 a un gran número de proteínas que están directa o indirectamente relacionadas en la regulación de la dinámica de actina (Wang et al., 1997). La localización de la proteína SORBS2 puede ser citoplasmática y nuclear (Hand and Eiden, 2005). SORBS2 puede unirse a diversas

proteínas del citoesqueleto, como es VCL (vinculina), una proteína que tiene un papel importante en la adhesión y migración celular. También se une a MLLT4 (también llamado AF6 o afadina) un componente de la membrana celular localizada en sitios especializados de contacto célula-célula (Kawabe et al., 1999).

El papel antitumoral de SORBS2 está relacionado con el control de la adhesión celular y con la disminución de la migración celular. En cáncer pancreático, la expresión de SORBS2 se encuentra disminuida, y esto contribuye al desarrollo de este tipo de tumor (Taieb et al., 2008). En epitelio cervical normal y en lesiones CIN3, se ha reportado la expresión de SORBS2, sin embargo, existe una reducción de su expresión o casi nula en cáncer cervical. Además se ha propuesto como un gen supresor de tumor para este tipo de cáncer (Backsch et al., 2011).

**GPR137 (G protein-Coupled Receptor 137)**. Los receptores acoplados a proteína G (GPCRs), son mediadores importantes de la transducción de señales y son considerados blancos farmacológicos. En el año 2003, se describió el descubrimiento de 7 nuevos genes humanos que codifican para GPCRs: GPR133, GPR134, GPR135, GPR136 y GPR137, mediante el uso de la base de datos del GeneBank genomic (homology screening), basado en las secuencias que codifican a los receptores conocidos acoplados a proteínas G (Vanti et al., 2003).

El gen de GPR137 se localiza en el cromosoma 11 en la posición q13.1 (11q13.1). Los transcritos de GPR137 se han encontrado en el sistema nervioso central, en el sistema endocrino, reproductivo, y en el sistema pulmonar, lo que indica que GPR137 está implicado en una variedad de procesos fisiológicos (Regard et al., 2008, Vanti et al., 2003).

GPR137 al igual que otras proteínas acopladas a receptor G, está involucrado en el desarrollo y progresión de cáncer. Se ha reportado su expresión elevada en cáncer de próstata, donde tienen un papel en la proliferación y migración (Ren et al., 2016). La eliminación del gen de GPR137 en líneas celulares de cáncer de páncreas, resulta en la inhibición de proliferación celular y la formación de colonias e induce la apoptosis a través de la sobre-regulación de la caspasa 3 (Cui et al., 2015). De la misma manera, en líneas celulares de carcinoma hepatocelular (HCC) la disminución de la expresión de GPR137 resulta en la inhibición del

crecimiento celular (Shao et al., 2015), mientras que en células de meduloblastoma inhibe la proliferación (Wang et al., 2015a).

# CAPÍTULO 1

## DNMT3B modulates the expression of cancer related genes and downregulates the expression of the gene VAV3 via methylation

## Correo de aceptación del artículo para publicación

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Dear Dr. HernÃ¡ndez,
Your manuscript entitled "DNMT3B modulates the expression of cancer-related genes and downregulates the expression of the gene VAV3 via methylation" has been reviewed. The tracking number for this manuscript is AJCR0039037R1.

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Best regards

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## Title Page

# DNMT3B modulates the expression of cancer-related genes and downregulates the expression of the gene VAV3 via methylation

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**Running title:** DNMT3B downregulates the expression of cancer-related genes

## Abstract
Altered promoter DNA methylation is one of the most important epigenetic abnormalities in human cancer. DNMT3B, *de novo* methytransferase, is clearly related to abnormal methylation of tumour suppressor genes, DNA repair genes and its overexpression contributes to oncogenic processes and tumorigenesis *in vivo*. The purpose of this study was to assess the effect of the overexpression of DNMT3B in HaCaT cells on global gene expression and on the methylation of selected genes to the identification of genes that can be target of DNMT3B. We found that the overexpression of DNMT3B in HaCaT cells, modulate the expression of genes related to cancer, downregulated the expression of 151 genes with CpG islands and downregulated the expression of the VAV3 gene via methylation of its promoter. These results highlight the importance of DNMT3B in gene expression and human cancer.

**Key Words:** methylation, *de novo* methyltransferase, overexpression of DNMT3B, cancer, cancer-related genes, VAV3, CpG island

# Main Text

## Introduction

Epigenetic and genetic alterations are common in the genesis and progression of various types human cancer. The abnormal expression of genes related to cell cycle, DNA repair, cellular metabolism and tumor suppressor are frequent defects that contribute to development of cancer [1]. Abnormal DNA methylation is one of the most important epigenetic factors directly involved in tumourigenesis, because methylation can induce repression of tumor suppressor genes or activation of oncogenes [2].

In human cancer the patterns of DNA methylation are altered: the overall level of DNA methylation is lower in normal cells than in cancer cells and the methylation of CpG islands of tumor suppressor and DNA repair is higher in cancer than normal cells [3]. DNA methylation at the 5' cytosine of CpG sites is catalyzed by DNA methyltranferases (DNMTs). The DNMT family includes three enzymes, DNMT1 responsible for maintaining pre-existing methylation patterns after DNA replication and DNMT3A and DNMT3B, *de novo* methyltransferases that are required to establish methylation during development and imprinting [4-5]. Genetic abnormalities and aberrant overexpression of DNMTs contribute to DNA hypermethylation in cancer [6-7]. Inhibition of these enzymes in cancer can decrease DNA methylation, reactivate silence genes and diminish tumorigenicity [8]. Furthermore, it has been showed that DNMT3B is overexpressed in cell lines of cancer and in several types of primary tumors [9-14]. In several words of cancer, it has has been reported that there is a positive correlation between DNMT3B expression and promoter DNA methylation [11, 13, 15-16]. Interestingly, DNMT3B contributes to oncogenic processes and tumorigenesis *in vivo* by gene-specific *de novo* methylation and transcriptional silencing [17]. Overexpression of DNMT3B protein significantly contributes to elevated methyltransferase activity and hypermethylation in breast cancer cells [13]. Although, the important role of DNMT3B in cancer development is clear, at present only a few genes have been identified as targets for transcriptional regulation by this enzyme [18-21].

Therefore, the purpose of this study was to assess the effect of the overexpression of DNMT3B in HaCaT cells on global gene expression and on the methylation of selected genes to the identification of genes that can be target of DNMT3B. We found that the overexpression of DNMT3B in HaCaT cells downregulated the expression of VAV3, SORBS2, and GPR137 genes by microarray and RT-qPCR and a clear increase in DNA methylation was detected in VAV3 promoter.

## Materials and methods

### Cell culture and cervical samples

The HaCaT (human skin keratinocyte), C-33A (cervical cancer), HeLa (cervical cancer), SiHa (cervical cancer), A549 (lung adenocarcinoma) and MCF-7 (breast adenocarcinoma) cells lines were obtained from American Type Culture Collection (ATCC, USA), cultured in DMEM and F-12 1:1, medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown at 37°C in 5% $CO_2$. The samples were collected at the Cancer Institute of the State of Guerrero located in southern Mexico. The population consisted of 25 healthy women

and 25 women with cervical cancer. The diagnosis of normal cervix was done by cytomorphological examination through conventional Papanicolaou test and cervical cancer by histological diagnosis, according to the classification system of the International Federation of Gynecology and Obstetrics (FIGO). All samples were obtained after the patients gave their informed consent and the Bioethics and Research Committee of the Cancer Institute of the State of Guerrero, Mexico, approved the study, which followed the ethical guidelines of the 2008 Helsinki Declaration.

### Transient transfection

Complementary DNA encoding DNMT3B was cloned into pcDNA3.1(+) plasmid (Invitrogent, Carlsbad, CA USA) to generate the pcDNA-DNMT3B expression plasmid that was confirmed by sequencing. The HaCaT cells (25 x $10^3$ cells, 6-well plates) were transfected with Lipofectamine 2000 Reagent (Invitrogent) according to the manufacturer's protocol. The cells were transfected with 3.5 µg of pcDNA-DNMT3B plasmid or empty vector pcDNA3.1(+) and after 48 h the cells were harvested for RNA and DNA extraction.

### RNA and DNA extraction

Total RNA was isolated and purified from the cell lines and cervical tissue with Direct-zol RNA MiniPrep (ZYMO Research, Invine, USA) according to the manufacturer's instructions including DNase I treatment. RNA integrity was determined by electrophoresis in a 1% agarose gel. Genomic DNA was extracted from the cells using a standard phenol chloroform method [22]. The concentration of RNA and DNA was evaluated by spectrophotometry using NanoDrop 2000c (Thermo Scientific, Wilmington, DE USA).

### Microarray analysis

H35K array was performed in Microarray Unit of Cellular Physiology Institute, UNAM, Mexico City. H35K contains 70-mer oligonucleotide probes representing 35764 human transcripts. Total RNA was extracted of HaCaT cells transfected with pcDNA-DNMT3B and of HaCaT cells transfected with pcDNA3.1(+) (empty vector). Equimolar concentrations of total RNA from of 3 independent experiments were mixed. Ten µg of RNA were used for cDNA synthesis and equal quantities of Cy3-labeled cDNA from control cells and Cy5-labeled cDNA from experimental cells were hibridized to the H35K array. Each hybridization was carried out in duplicate. Array signal intensities were analyzed with ScanArray 4000 from Packard BioChips. Microarray data analysis, background correction, normalization and selection of differentially expressed genes were performed with GenArise software (http://www.ifc.unam.mx/genarise/). Differentially expressed genes were selected according to the Z-score value [23]. Differential expressed genes were considered upregulated when Z-score > 1.5 standard deviation or downregulated when Z-score < 1.5 standard deviation.

## Bioinformatics analysis

Gene ontology (GO) analysis of the differentially expressed genes was performed with PANTHER (http://www.pantherdb.org/) and according to the program an enrichment score of $P < 0.05$ was considered as significant. For promoter prediction we considered 3000 pb (-2000 pb to +1000 pb) relative to ATG using the ExPASy Bioinformatics Resource Portal (http://www.expasy.org/genomics). For CpG island prediction the criteria was regions >200 bp with a GC content ≥50% with an observed CpG/expected CpG >0.6 [24]. CpG islands prediction was done using the Methprimer Program (http://www.urogene.org/methprimer/). The prediction of transcription factors that can bind to VAV3 promoter was done with CONSITE database (http://consite.genereg.net/).

## RT-qPCR

One hundred ng of total RNA were used in each RT-qPCR assay. Reverse transcription and quantitative PCR were performed with KAPA SYBR FAST One-Step qRT-PCR kit (Kapa Biosystems, Boston, Massachusetts, USA), according to the manufacturer's protocol. In all cases, the conditions of reverse transcription and amplifications were: 30 s at 37 °C, 42 °C for 5 min and 95 °C for 5 min; 40 cycles of amplification: 5 s at 95°C, 30 s at 60 °C and 30 s at 72 °C; melt curve: 15 s at 95 °C, 1 min at 60 °C and 15 s 95 °C. The reactions were done in Real Time ABI-PRISM 7500 SDS (Applied Biosystems, Foster City, CA). Data were normalized using GADPH as an internal control and relative expression differences were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers sequences are shown in Table 1.

## Methylation-specific PCR (MSP) and Bisulfite sequencing (BSP)

For MSP, 1 µg of DNA was treated with sodium bisulfite using the EpiTect Bisulfite kit (QUIAGEN, Hilden, Germany) according to the manufacturer's instructions. MSP primer sequences are shown in Table 1. MSP was performed in a total of 10 µL, containing 1 µL of bisulfite-treated DNA, 250 nM of each primers and AmpliTaq Gold360 Master Mix (Applied Biosystems) and under the following amplification conditions: denaturation 95 °C for 10 min, 40 cycles of amplification: 30 s at 95°C, 30 s at 60 °C and 30 s at 72 °C, and a final extension of 72 °C for 10 min. Bisulfite sequencing was done for VAV3, SORBS2, and GPR137 genes. The promoters of this genes were divided into two regions to facilitate the methylation analysis. One hundred ng of bisulfite-treated DNA was used as a template, and PCR was performed using specific primers (Table 1). The reactions were done in Eppendorf Mastercycler EP Gradient 96 Thermal cycler (Applied Biosystems). The PCR products were gel purified and cloned into the pJET1.2/blunt vector (Thermo Scientific). Five independent clones were subjected to automated sequencing (ABI Prism 310 Genetic Analyzer (Applied Biosystems).

## Statistical analysis

The data are shown as mean ± standard deviation. The *P* value was determined using Student´s *t*-test. *P* values below 0.05 were considered statistically significant.

## Results

DNMT3B has an important role in aberrant DNA methylation to repress transcription. To identify downregulated genes by DNMT3B, we overexpressed DNMT3B in the HaCaT cell line, and H35K microarray that interrogated 35764 genes was used to identify changes in gene expression. We found 1085 downregulated genes, 1741 upregulated genes and 32938 unchanged genes (Figure 1A). To gain insights into the biological processes where 1085 downregulated genes are implicated, we carried out a gene ontology (GO) analysis using Protein Analysis Through Evolutionary Relationships (PANTHER). This analysis revealed that an important part of the 1085 downregulated genes are involved in the immune system, development processes, cell communication, cellular processes and metabolic processes (Figure 1B). The GO analysis for the 1741 upregulated genes is shown in supplementary Figure 1.

The 1085 downregulated genes were classified according to Z-score value (Figure 2A). We narrowed down this group of genes by the selection of gene subsets with Z-scores of -2 to -6.8 (252 genes). Hypermethylation of CpG islands found within promoters is clearly related to transcriptional repression. Therefore, to relate the 252 downregulated genes with the methylation of its promoter by overexpression DNMT3B, we used MethPrimer to prediction of CpG islands for 252 genes. We found 151 genes with CpG islands, 73 genes without CpG islands and 28 genes with absent data (Figure 2B). To know the biological processes where 151 genes with CpG islands are involved, we carried out GO analysis. We found that some of these genes are implicated in molecular and cellular processes altered in cancer such as adhesion, apoptosis, response to stimulus, development, biological regulation and metabolic processes (Figure 2C). Among the 151 genes with CpG islands, we find genes with previous reports of abnormal methylation in several types human tumors, many genes putative or tumor suppressor and genes related with cancer. The complete list of 151 genes with CpG islands is shown in supplementary Table 1.

To validate the results of the microarray, we analyzed the level of expression of 10 genes by RT-qPCR. These 10 genes were selected for further validation because 1) they were downregulated by overexpression of DNMT3B, 2) they have CpG islands and 3) they are involved in regulating important molecular and cellular functions which are disrupted in cancer. The function of 10 genes is shown in supplementary Table 2. The level of expression of 7 genes was consistent with data from microarray analysis and inconsistent in three genes (Figure 3). The analysis by RT-qPCR showed that expression levels of SORBS2, VAV3 and GPR137 mRNAs were significantly downregulated by the overexpression of DNMT3B.

To clarify whether downregulation of VAV3, SORBS2, and GPR137 is mediated by DNA hypermethylation in overexpression of DNMT3B HaCaT cells, we analyzed the methylation status of its promoters by using methylation-specific PCR (MSP) and bisulfite conversion and sequencing. For the VAV3 gene, its CpG island spanning from -599 pb to +20 pb of the transcription start site, within of this region we found 95 CpGs sites (Figure 4A). No obvious methylation changes were observed between HaCaT cells and HaCaT cells with overexpression of DNMT3B by MSP analysis (Figure 4B). To make a more detailed analysis of methylation status, we analyzed the methylation in the 95

CpGs sites of the VAV3 promoter. We found two small, more densely methylated regions (15, 16, 17, 18, 19 and 21 CpG sites of region 1 and 52, 53, 54, 55, 56, 57, 58 and 59 CpG sites of region 2) of the VAV3 promoter in HaCaT cells with overexpression of DNMT3B in comparison with HaCaT cells (Figure 4C). These results suggest that the overexpression of DNMT3B in HaCaT cells probably has a role in the methylation of the VAV3 promoter. The MSP and bisulfite conversion and sequencing analysis was done for SORBS2, and GPR137 genes but no methylation changes were observed between HaCaT cells with overexpression of DNMT3B and HaCaT cells (supplementary Figure 2 and 3).

Finally, to correlate our results with what occurs in human cancer, we analyzed the expression of DNMT3B in cervical cancer samples and normal cervical tissue. As well as DNMT3B, VAV3, SORBS2 and GPR137 expression in cervical, lung and breast cancer cell lines. RT-qPCR analysis showed that mRNA level of DNMT3B in cervical cancer samples was significantly higher that in normal tissue (Figure 5A). In general, in the analyzed cell lines, we found overexpression of DNMT3B and low levels VAV3, SORBS2 and GPR137 (Figure 5B). These results suggest that overexpression of DNMT3B can be a common event in human cancer and expression of VAV3, SORBS2 and GPR137 could be regulated by DNMT3B.

## Discussion

DNMT3B overexpression and abnormal methylation of tumour suppressor and DNA repair genes are common alterations in several types of human cancer [6, 25]. There is evidence indicating the involvement of DNMT3B in the initiation and progression of cancer [20, 26]. In addition DNMT3B is clearly related to the abnormal methylation in cancer [21, 27]. Although only 5 genes have been identified as targets for transcriptional repression by DNMT3B [18-21].

In this work the overexpression of DNMT3B in HaCaT cells downregulated 151 genes with CpG islands. This result suggests that the downregulated genes could be result from the methylation of its promoter by DNMT3B overexpression. In this sense, it has been reported that DNMT3B preferably to methylate CpG-dense promoter regions and is excluded from active promoters [28]. Also, downregulation or repression by methylation requires promoters with high methylated-cytocines [29-31]. In initiation and progression of cancer, DNMT3B has directly or indirectly been associated with abnormal expression and methylation [8, 26-27]. An similar scenario it could be also seen in our study in which of the downregulated 151 genes by DNMT3B were found 22 genes with previous reported of abnormal methylation in several types of human cancer, 9 reported as putative or tumor suppressors genes and 61 genes related to many aspects of human cancer.

The overexpression of DNMT3B in HaCaT cells, downregulated the expression of VAV3, SORBS2, and GPR137 genes by RT-qPCR, but a clear increase in DNA methylation was only detected in the VAV3 promoter. Therefore it is possible that the VAV3 gene is regulated by DNMT3B via methylation of its promoter. VAV3 is a guanine nucleotide

exchange factor involved in the regulation of Rho GTPases and in several cellular processes, including regulation of cytoskeleton organization, cell transformation and oncogenesis [32-34]. In addition, abnormal methylation of the VAV3 promoter has been reported in breast cancer cell lines and in gastric cancer the methylation of its promoter is considered as a marker to estimate the fraction of cancer cells in primary gastric cancer [35-36]. On the other hand, we detected methylation of the VAV3 promoter in HaCaT cells without overexpression of DNMT3B. Although this result is unexpected, previously methylation of the VAV3 promoter in normal cells of the gastric mucosa has been reported [36]. By *in silico* analysis with CONSITE we detected that the transcription factors: Sp1, AP2 alpha, MZF, E2F, Hen-1 and Thing1-E4 can bind to localized sites in the more densely methylated regions of the VAV3 promoter. It is well known that the methylation of CpG in the Sp1 binding site generally interferes with its binding and can affect the transcription [37-38]. The E2F transcription factor, does not bind DNA when their site recognition is methylated [39]. To some promoters AP2 alpha can act as a suppressor for Sp1 binding, also the AP2 alpha binding to DNA may initiate transcriptional silencing by recruiting of DNMTs [40-41]. Therefore it is possible that the methylation of binding sites Sp1, AP2 alpha and E2F located in the two more densely methylated regions of VAV3 promoter can inhibit its binding and its subsequent transcriptional activation. This event could explain the expression decrease of the VAV3 gene in HaCaT cells with overexpression of DNMT3B.

The overexpression of DNMT3B in HaCaT cells, downregulates the expression of SORBS2 and GPR137 genes, but the methylation of its promoters do not increase. SORBS2 is a scaffold protein involved in the assembly of signaling complexes in stress fibers and actin cytoskeleton [42-43]. This gene is considered as putative tumour suppressor and although there is evidence of the loss or decrease of its expression in cervical and pancreatic cancer [44-45], there is no evidence that this is due to promoter methylation. GPR137 is an integral membrane protein that belongs to the GPR137 family of cell mediators of signal transduction [46-47]. Although the role of GPR137 in cancer is little known, several reports indicate that this gene is important a regulator of cell growth, apoptosis, invasion and migration in different types of human cancer [48-52]. Similar to SORBS2 there are no reports of abnormal methylation of the GPR137 promoter in human cancer. It is therefore likely that additional events are causing the downregulation the expression of SORBS2 and GPR137 genes. For example, methylation-independent repressor activities of DNMT3B [53].

In the current study, we found overexpression of DNMT3B in cervical cancer and various cancer cell lines. This event has been previously reported in various types of human cancer [8-9, 13]. We also reported overexpression of DNMT3B and low levels of VAV3, SORBS2 and GPR137 in cervical, lung and breast cancer cell lines. This could indicate that the our findings in the DNMT3B overexpression in HaCaT cells model also occur in primary human tumors and human cancer cell lines.

In conclusion, our results suggest that the overexpression of DNMT3B in HaCaT cells, modulate the expression of genes related to cancer, downregulate the expression of 151 genes with CpG islands and downregulate the expression of the VAV3 gene via

methylation of its promoter. These findings highlight the importance of DNMT3B in the gene expression and human cancer.

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**Table 1.** Primer sequences used in this study

| Gene | Sequence | Tm °C |
|---|---|---|
| | **RT-qPCR** | |
| MSH2 | F5´-TTCATGGCTGAAATGTTGGA<br>R5´-ATGCTAACCCAAATCCATCG | 59 |
| NSD1 | F5´-TGAAGGCAGACATCAATTCG<br>R5´-CCAACTTGATTGAACCAGGAA | 55 |
| SORBS2 | F5´-AAGCACAGCCTGCAAGACCA<br>R5´-TGGGGTATTGGAGGGTCAGG | 60 |
| ARHGAP29 | F5´-TTAGAGGATGTTGTACGCC<br>R5´-TTCGATGAAAGTCTCCTGG | 58 |
| VAV3 | F5´-ACAAGGAGCCAGAACATTCAG<br>R5´-TTGCACAGAAGTCATACCGAG | 58 |
| GPR137 | F5´-TCAGCTATCAGACGGTGTTC<br>R5´-AGCAGTAGAGAAGCCAGAAG | 52 |
| C1ORF201 | F5´-CTTGTGAAGCAGTCGCCAAATACAT<br>F5´-CACGATCTCATACTGACCAGGACCT | 58 |
| THSD1 | F5´-GGAGGCCAACACCAATCAGA<br>R5´-CAGTAGTCACCAGCCTCCTT | 59 |
| ST6GALNAC2 | F5´-GGGTCGTTCTTCTGGCTGCT<br>R5´-TGATGTGGTGTCCCTGGCTC | 59 |
| MSX1 | F5´-CCAGAAGATGCGCTCGTCAA<br>R5´-TCGTCTTGTGTTTGCGGAGG | 59 |
| GADPH | F5´-CCGGGAAACTGTGGCGTGATGG<br>R5'-AGGTGGAGGAGTGGGTGTCGCTGTT | 60 |
| | **MSP** | |
| VAV3-R1 M | F5´-GTTTTGGGGGATTTTATCGTATTAT<br>R5´-GACCCGCCACTAAACATACCCAAC | 58 |
| VAV3-R1 U | F5´-TGGGGGATTTTATTGTATTATAGTA<br>R5´-AACCCACCACTAAACATACCCAACA | 55 |
| VAV3-R2 M | F5´GGCGTTGGAGTCGGAAGTTTGTG<br>R5´-CACTACTTCCACGACTCCATACC | 60 |
| VAV3-R2 U | F5´-GGTGTTGGAGTTGGAAGTTTGTGT<br>R5´-CACACACTACTTCCACAACTCCATACC | 59 |
| SORBS2-R1 M | F5´-ATAATAAAAGAATAAATTTAGGTCGGG<br>R5´-CTATCGCCCAAACTAAAATACAAT | 58 |
| SORBS2-R1 U | F5´-TATAATAAAAGAATAAATTTAGGTTGGG<br>R5´- AAAATAAAATCTCACTCTATCAC | 54 |
| SORBS2-R2 M | F5´-GGGAATTATGTGTTAATTTAATTCG<br>R5´-AAATCATAAATACTAAACGCTCC | 52 |
| SORBS2-R2 U | F5´-GGAATTATGTGTTAATTTAATTTGATG<br>R5´-ATAAAATCATAAATACTAAACACTCC | 56 |
| | **BSP** | |
| VAV3-R1 | F5´-AGGGGGTTTTGGGGGATTTTAT<br>R5´-CCACTAAACATACCCAACA | 56 |
| VAV3-R2 | F5´-GGCGTTGGAGTCGGAAGTTTGTG<br>R5´-CACTACTTCCACGACTCCATACC | 60 |
| SORBS2-R1 | F5´-AGTTATAAAATTTTGATTGGTTGA<br>F5´-AACCTACAAACTTACTCTAAATCCTAT | 58 |
| SORBS2-R2 | F5´-GGAATGATGTTTATAGGGAATTATGTG<br>F5´-CCCTAAAAATAAAATCATAAATACTAAA | 59 |
| GPR137-R1 | F5´-GGGGGTATTGGAGATAAGGAAAGG<br>F5´-CTCCTCTCTCCTATACCCAAATC | 59 |
| GPR137-R2 | F5´-TTTTTTTTTTTTGAGGTTGGAG<br>F5´-CAAACCCCTCACTCAAAAACA | 59 |

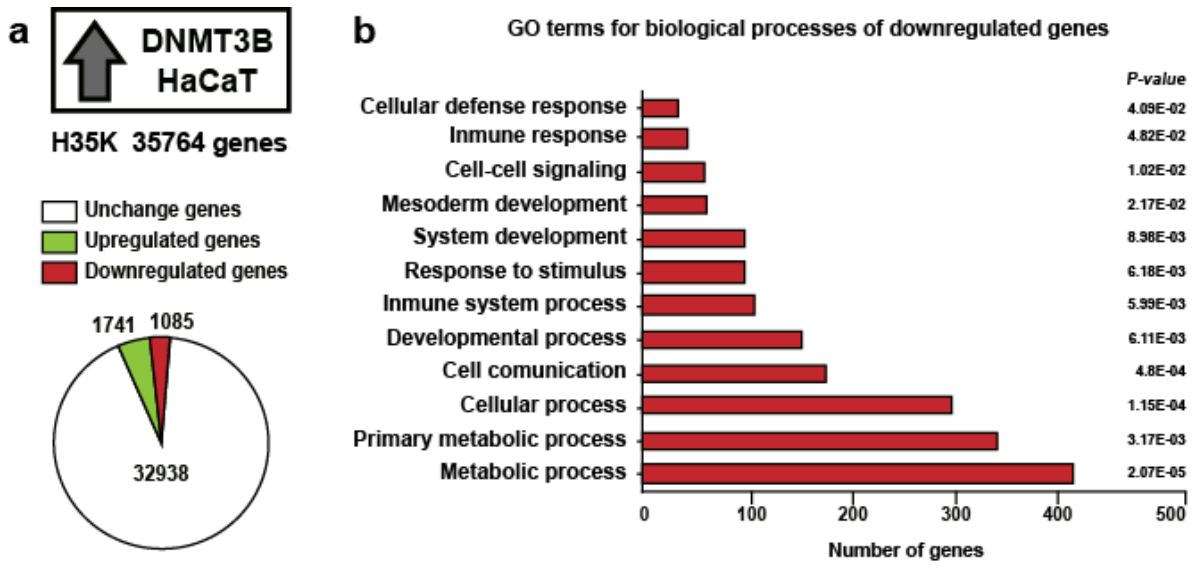
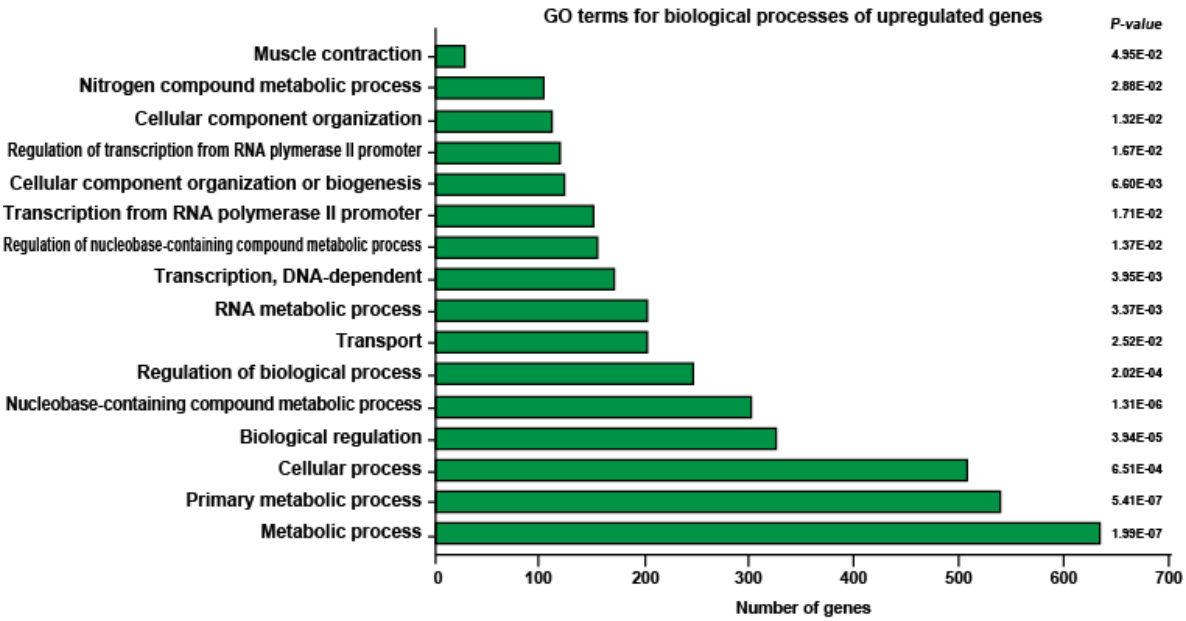



**Figure 1.** Gene ontology analysis of downregulated genes by overexpression of DNMT3B in HaCaT cells. a) We used H35K array of 35764 genes, the graph shows the number of genes that change their expression by overexpression of DNMT3B. b) Gene ontology (GO) analysis for downregulated genes by overexpression of DNMT3B.




**Supplementary Figure 1.** Gene ontology (GO) analysis for upregulated genes by overexpression of DNMT3B in HaCaT cells.

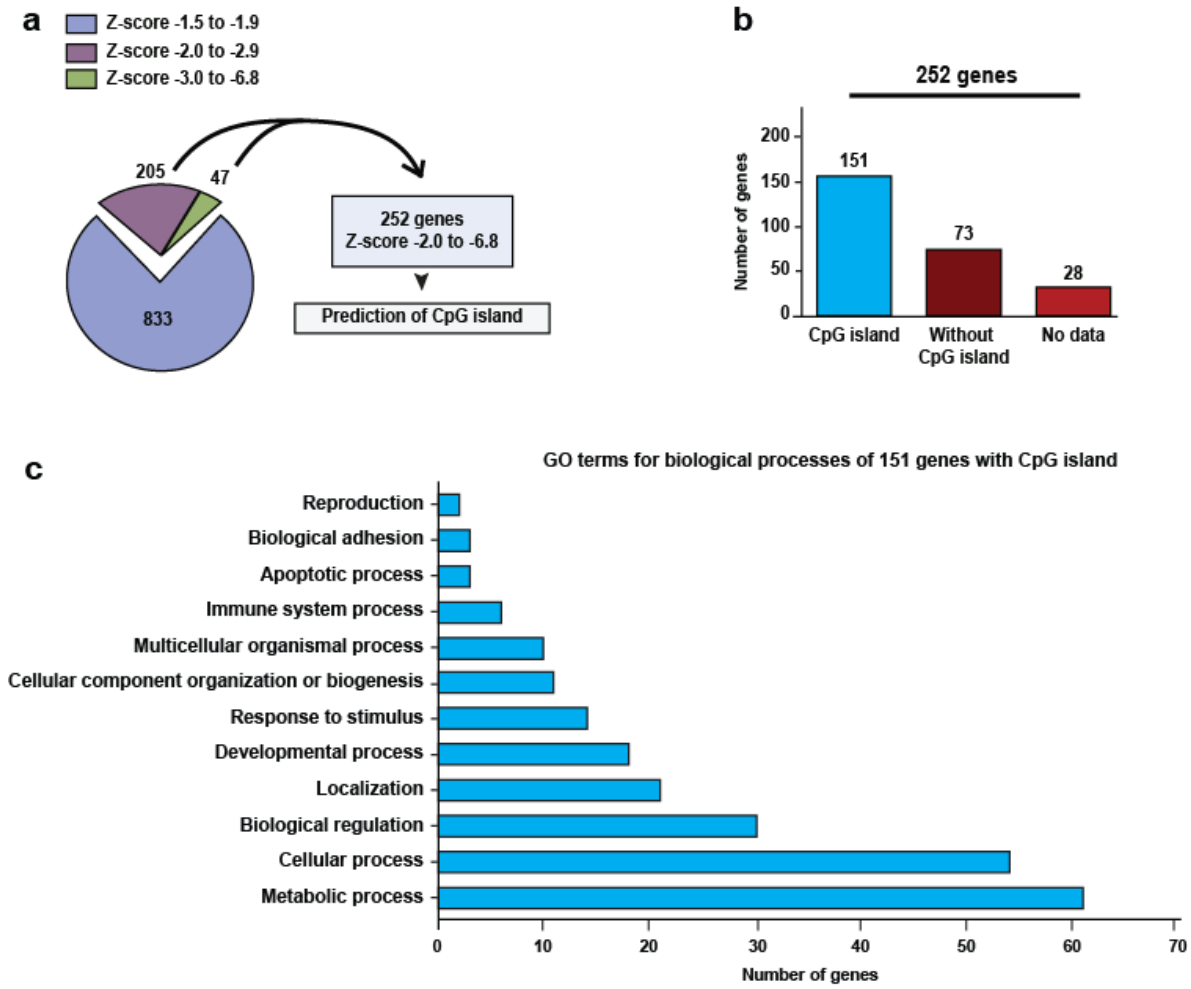



**Figure 2.** Prediction of CpG island in downregulated genes by overexpression of DNMT3B in HaCaT cells. a) Classification of downregulated genes according to Z-score value, the graph shows the number of genes for each Z-score range. b) Number of genes with and without CpG island. c) Gene ontology (GO) analysis for 151 genes with CpG island.

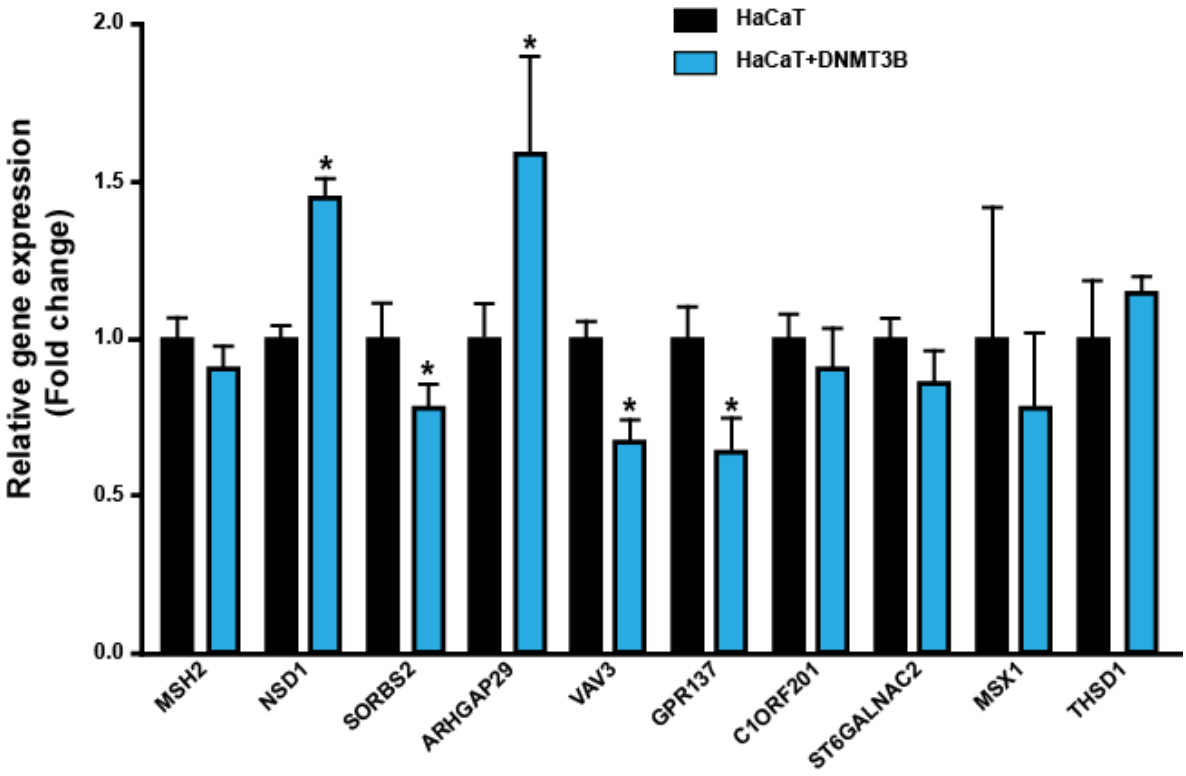



**Figure 3.** Validation of microarray data by RT-qPCR. mRNA quantification of 10 genes in HaCaT cells with overexpression of DNMT3B and control HaCaT cells. The bars represent the mean ± standard deviation from at least three independent experiments. **P* < 0.05

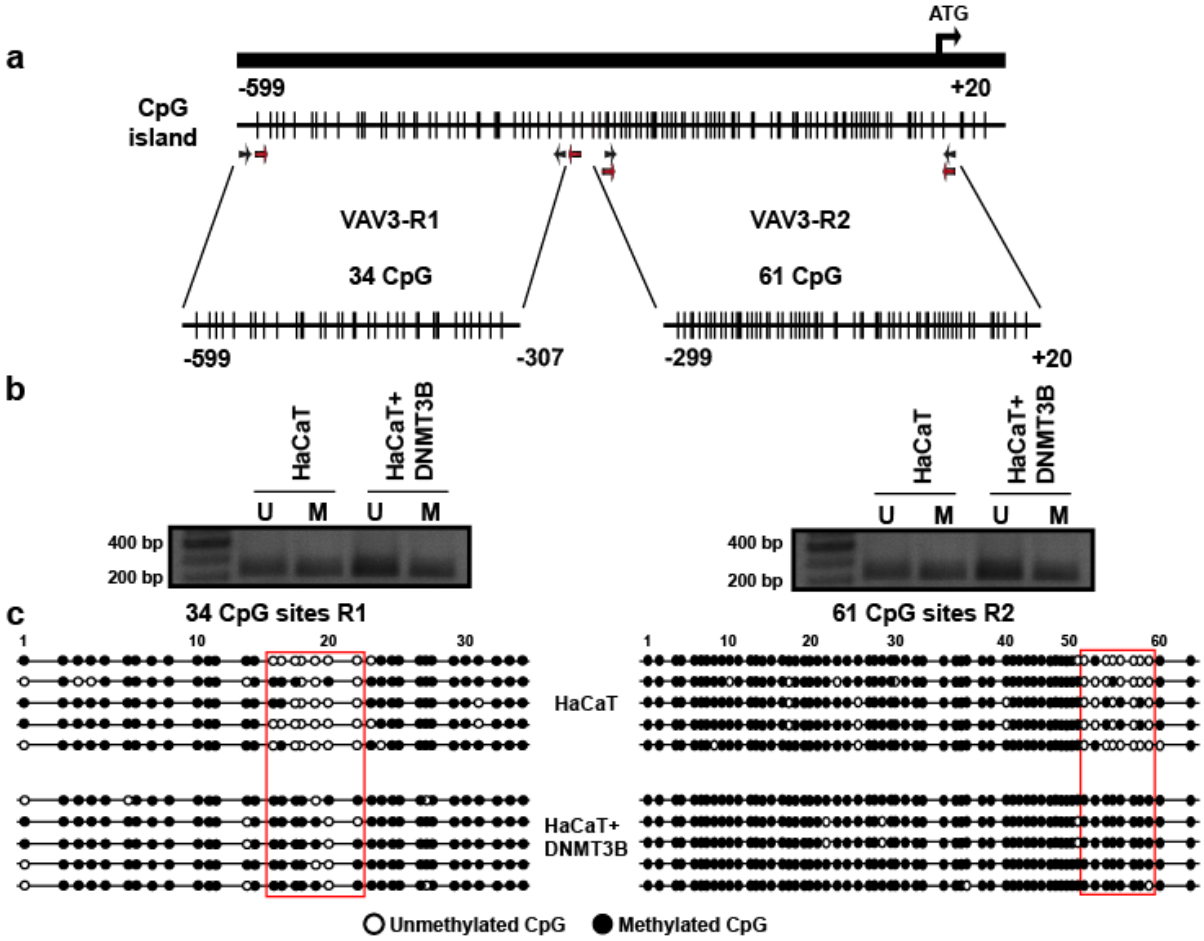
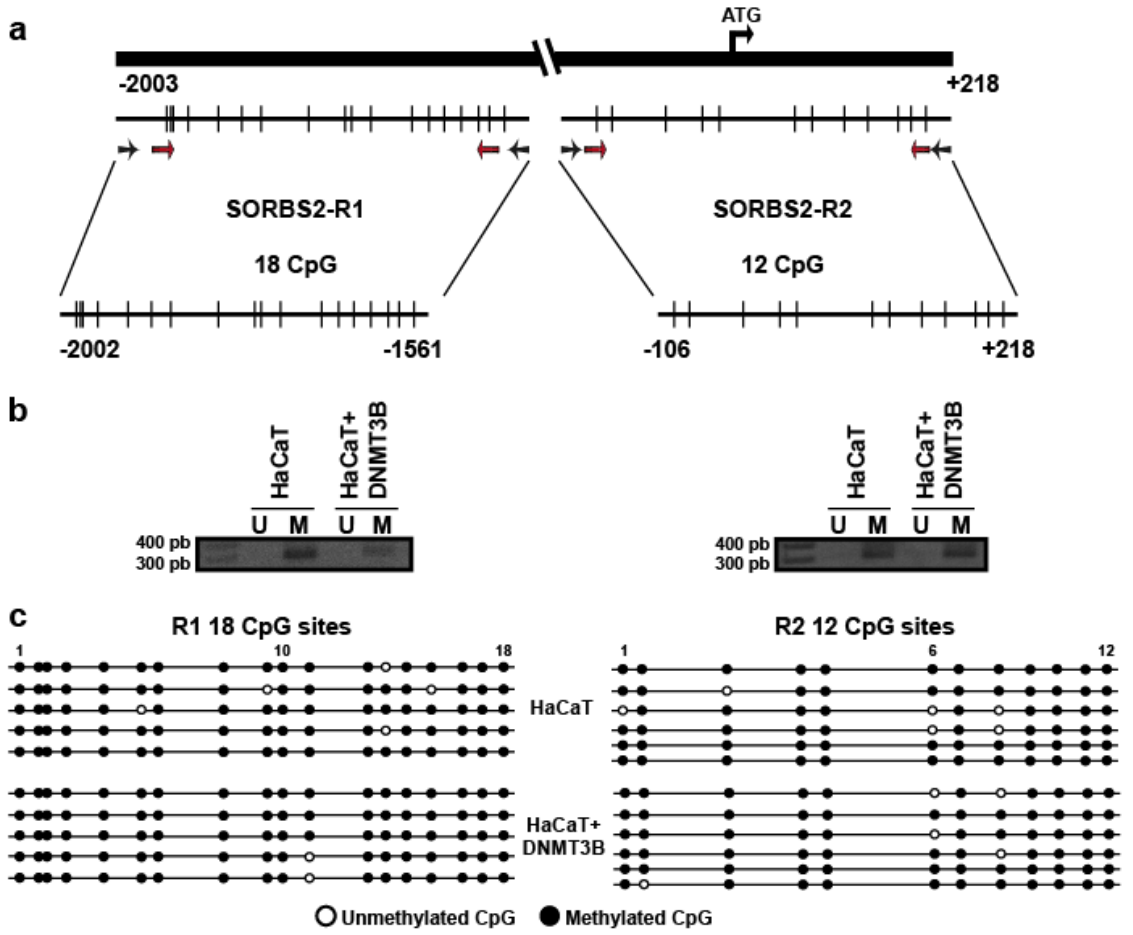
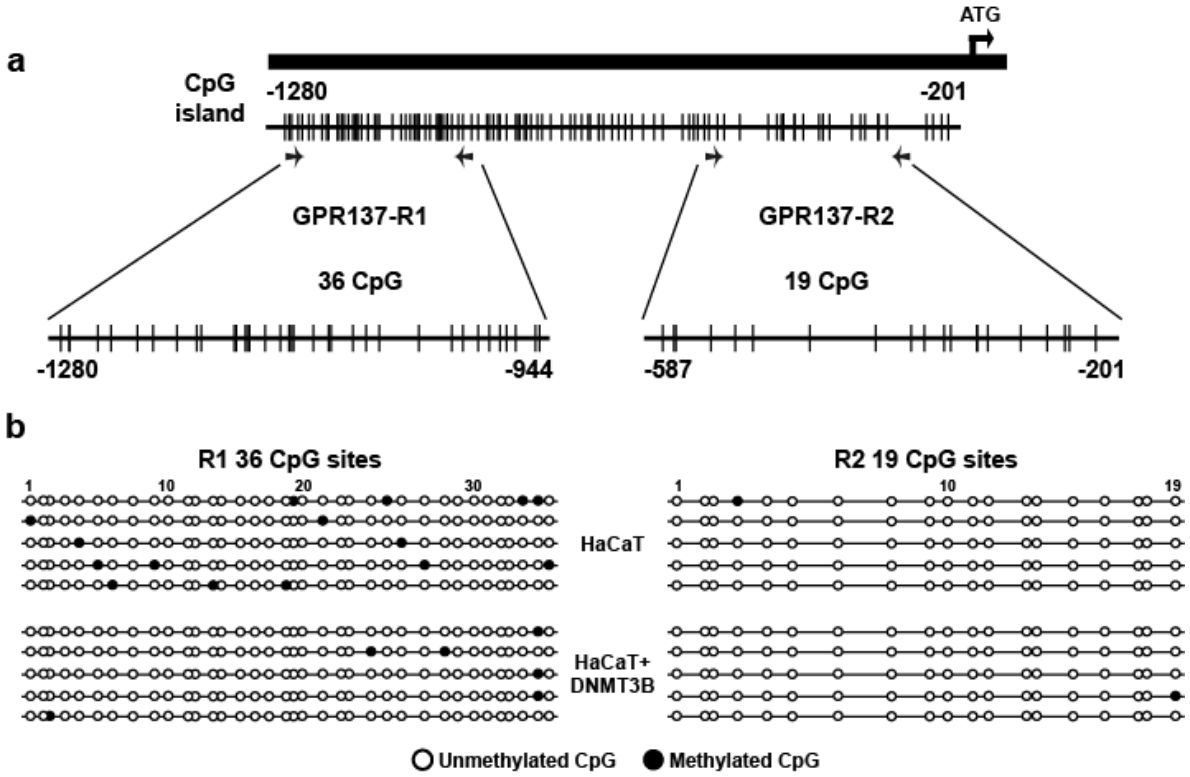



**Figure 4.** Methylation analysis of VAV3 promoter in HaCaT cells. a) Schematic representation of the CpG island and CpG sites in the VAV3 promoter. For methylation analysis the VAV3 promoter was divided into 2 regions: R1 -599 to -307 with 34 CpGs and R2 -299 to +20 with 61 CpGs, the positions are relative to the transcription start site. The primers for MSP and BSP are indicated by black and red arrows, respectively. Each CpG site is represented by a vertical bar. b) The methylation status of the VAV3 promoter (R1 and R2) was determined by MSP in HaCaT cells with overexpression of DNMT3B and control HaCaT cells. U showed unmethylation-specific primer amplification, M showed methylation-specific primer amplification. c) BSP analysis of the VAV3 promoter (R1 and R2) in HaCaT cells with overexpression of DNMT3B and control HaCaT cells. Black circles represent methylated CpG site and white circles represent unmethylated CpG site. The red box shows the two regions more densely methylated by overexpression of DNMT3B.




**Supplementary Figure 2.** Methylation analysis of SORBS2 promoter in HaCaT cells. a) Schematic representation of the CpG island and CpG sites in the SORBS2 promoter. For methylation analysis the SORBS2 promoter was divided into 2 regions: R1 -2002 to -1561 with 18 CpGs and R2 -106 to +218 with 12 CpGs, the positions are relative to the transcription start site. The primers for MSP and BSP are indicated by black and red arrows, respectively. Each CpG site is represented by a vertical bar. b) The methylation status of the SORBS2 promoter (R1 and R2) was determined by MSP in HaCaT cells with overexpression of DNMT3B and control HaCaT cells. U showed unmethylation-specific primer amplification, M showed methylation-specific primer amplification. c) BSP analysis of the SORBS2 promoter (R1 and R2) in HaCaT cells with overexpression of DNMT3B and control HaCaT cells. Black circles represent methylated CpG sites and white circles represent unmethylated CpG sites.




**Supplementary Figure 3.** Methylation analysis of GPR137 promoter in HaCaT cells. a) Schematic representation of the CpG island and CpG sites in the GPR137 promoter. For methylation analysis the GPR137 promoter was divided into 2 regions: R1 -1280 to -944 with 36 CpGs and R2 -587 to -201 with 19 CpGs, the positions are relative to the transcription start site. The primers for BSP are indicated by red arrows. Each CpG site is represented by a vertical bar. b) BSP analysis of the GPR137 promoter (R1 and R2) in HaCaT cells with overexpression of DNMT3B and control HaCaT cells. Black circles represent methylated CpG site and white circles represent unmethylated CpG site.

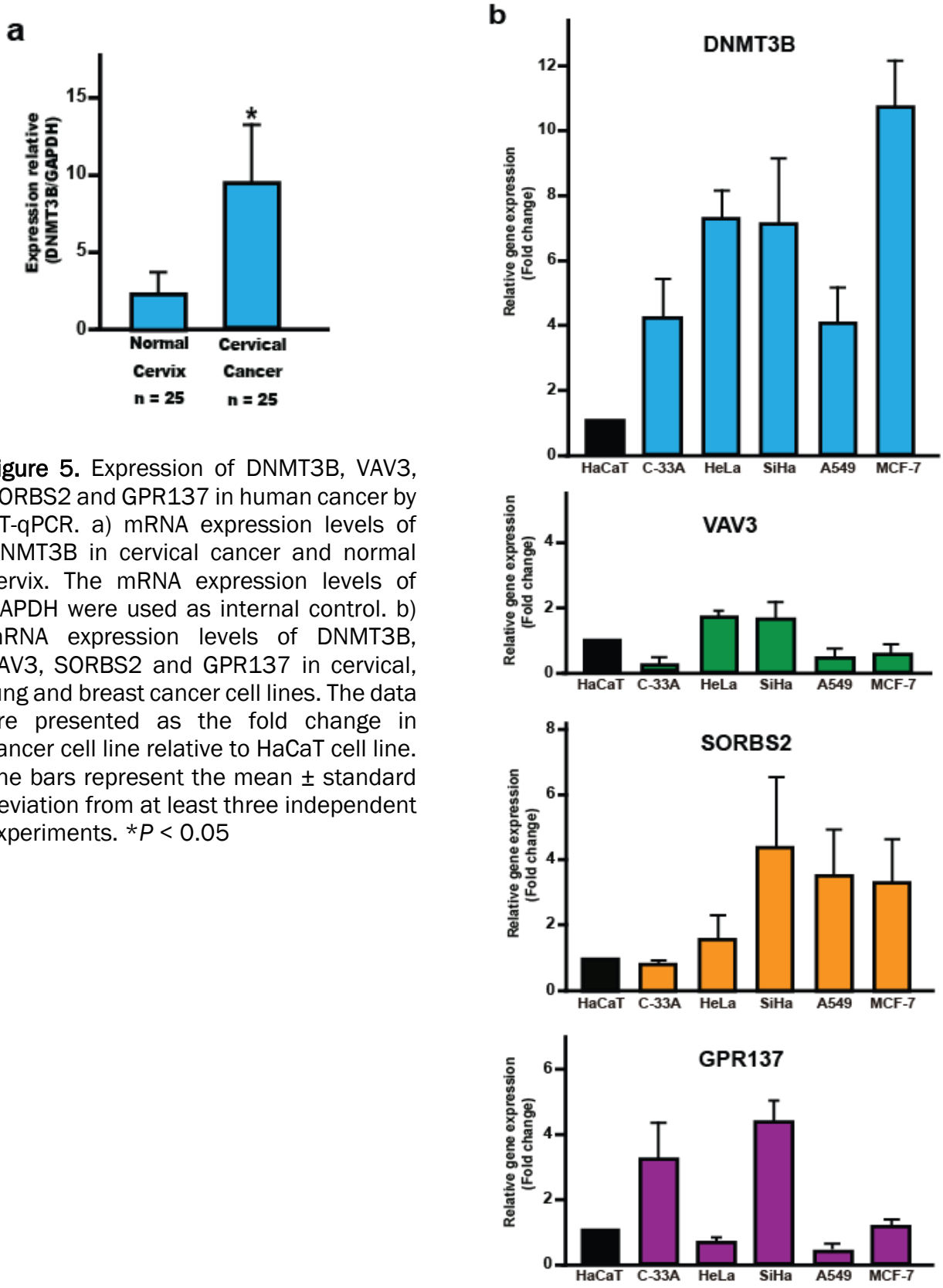



**Figure 5.** Expression of DNMT3B, VAV3, SORBS2 and GPR137 in human cancer by RT-qPCR. a) mRNA expression levels of DNMT3B in cervical cancer and normal cervix. The mRNA expression levels of GAPDH were used as internal control. b) mRNA expression levels of DNMT3B, VAV3, SORBS2 and GPR137 in cervical, lung and breast cancer cell lines. The data are presented as the fold change in cancer cell line relative to HaCaT cell line. The bars represent the mean ± standard deviation from at least three independent experiments. *$P < 0.05$

| **Supplementary Table 1.** Genes with CpG island downregulated by overexpression of DNMT3B in HaCaT cell | | | |
|---|---|---|---|
| **Gene-ID** | **Gene symbol** | **Gene name** | **Epigenetic evidence** |
| ENSG00000106477 | TSGA14 | Centrosomal protein 41kDa | Methylated in Ewing sarcoma (ES) cell lines and primary ES [1]. |
| ENSG00000134215 | VAV3 | VAV3 guanine nucleotide exchange factor | Methylated in breast cancer cell lines [2]. |
| ENSG00000196263 | ZNF471 | Zinc finger protein 471 | Methylated in colorectal cancer [3]. |
| ENSG00000163132 | MSX1 | Msh homeobox 1 | Methylated in leukemia (T-ALL, T-linage leukemia) [4], and testicular cancer [5]. Furthermore, MSX1 is a repressor of cell cycle in human ovarian cancer cells [6]. Downregulated in cervical cancer tissue and cervical cell lines [7]. |
| ENSG00000095002 | MSH2 | MutS homolog 2 | Methylated in hepatocellular carcinoma [8], and Lynch Syndrome tumors [9]. |
| ENSG00000165671 | NSD1 | Nuclear receptor binding SET domain protein 1 | Methylated in human neuroblastoma and glioma cells [10]. |
| ENSG00000170558 | CDH2 | Cadherin 2, type 1, N-cadherin | Methylated in primary gastric cancer, gastric cancer cell lines [11], and colon cancer [12]. |
| ENSG00000112541 | PDE10A | Phosphodiesterase 10A | Methylated in colorectal cancer [13]. |
| ENSG00000136158 | SPRY2 | Sprout RTK signaling antagonist 2 | Methylated in invasive prostate cancer cell lines (CaP) [14]. |
| ENSG00000197579 | TOPORS | Topoisomerase I binding, arginine/serine-rich E3, ubiquitin protein ligase | Methylated in colon adenocarcinoma [15]. |
| ENSG00000183044 | ABAT | 4-aminobutyrate aminotransferase | Methylated in myelodysplastic syndrome [16], and glioblastoma [17]. |
| ENSG00000162496 | DHRS3 | Dehydrogenase/reductase (SDR family) member 3 | Methylated in neuroblastoma [18], and melanoma cell lines [19]. |
| ENSG00000165325 | CCDC67 | Coiled-coil domain containing 67 | Methylated in gastric cancer [20]. |
| ENSG00000134202 | GSTM3 | Glutathione S-transferase mu 3 | Methylated in Barrett´s adenocarcinoma (BACs) samples [21]. |
| ENSG00000116667 | C1orf21 | Chromosome 1 open reading frame 21 | Methylated in squamous cell carcinoma (SCC) [22]. |
| ENSG00000137962 | ARHGAP29 | Rho GTPase activating protein 29 | Methylated in mantle cell lymphomas (MCL) cell lines and primary MCL samples [23]. |
| ENSG00000147889 | CDKN2A | Cyclin-dependent kinase inhibitor 2A | Methylated in cervical cancer [24, 25], in patients with non-invasive urinary bladder [26]. |
| ENSG00000108753 | TCF2 | HNF1B homeobox B | Methylated in ovarian cancer cell lines and primary ovarian cancers [27]. |
| ENSG00000116754 | SFRS11 | Serine/arginine-rich splicing factor 11 | Xenoestrogen bisphenol A (BPA) induce methylation of SFRS11 gene in human breast epithelial cells [28]. |
| ENSG00000172175 | MALT1 | MALT1 paracaspase | Methylated in oral carcinoma [29]. |
| ENSG00000113569 | NUP155 | Nucleoporin 155kDa | Methylation of NUP155 gene has been associated with breast cancer risk and is considered an epimarker in this type of cancer [30]. |
| ENSG00000136114 | THSD1 | Thrombospondin, type I, domain containing 1 | Methylated in colorectal cancer [31], and esophageal squamous cell carcinoma (ESCCC) [32]. |
| ENSG00000159346 | ADIPOR1 | Adiponectin receptor 1 | Methylated in overweight children [33]. |

| | | | |
|---|---|---|---|
| ENSG00000163702 | IL17RC | Interleukin 17 receptor C | Hipomethylated in age related macular degeneration (AMD) patients; therefore, suggesting that the DNA methylation pattern and expression of IL17RC may potentially serve as a biomarker for diagnosis of AMD [34]. |
| ENSG00000143194 | MAEL | Maelstrom spermatogenic transposon silencer | Hypomethylated in colorectal cancer [35]. |
| **Gene-ID** | **Gene symbol** | **Gene** | **Tumor suppressor evidence** |
| ENSG00000107968 | MAP3K8 | Mitogen-activated protein kinase kinase kinase 8 | Is a tumor suppressor in lung cancer [36]. |
| ENSG00000125347 | IRF1 | Interferon regulatory factor 1 | IRF1 acts as a tumor suppressor in breast cancer [37]. |
| ENSG00000070731 | ST6GALNAC2 | ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2 | ST6GALNAC2 acts as a breast cancer metastasis suppressor [38]. |
| ENSG00000136158 | SPRY2 | Sprout RTK signaling antagonist 2 | Is proposed as a potential tumor suppressor in prostate cancer [39]. |
| ENSG00000197579 | TOPORS | Topoisomerase I binding, arginine/serine-rich E3, ubiquitin protein ligase | Is possibly a tumor suppressor in colon adenocarcinoma [15, 40]. |
| ENSG00000165325 | CCDC67 | Coiled-coil domain containing 67 | Is a putative tumor suppressor gene in gastric cancer [20]. |
| ENSG00000137962 | ARHGAP29 | Rho GTPase activating protein 29 | Is a novel candidate tumor suppressor in mantle cell lymphomas [23]. |
| ENSG00000136114 | THSD1 | Thrombospondin, type I, domain containing 1 | Is considered a candidate tumor suppressor in esophageal squamous cell carcinoma [32]. |
| ENSG00000080839 | RBL1 | Retinoblastoma-like 1 | RBL1 or p70 can suppress the cell growth in Saos-2 and C-33A cells. The growth suppression effect of p70 is cell-type and cell-cycle stage dependent [41]; on the other hand, RBL1 is downregulated in gliomas and it can act as tumor suppressor [42]. |
| **Gen-ID** | **Gene symbol** | **Gene name** | **Cancer involvement** |
| ENSG00000173068 | BNC2 | Basonuclin 2 | Lower BNC2 expression has been demonstrated in epithelial ovarian cancer (EOC) cell cultures compared to normal ovarian cell lines [43].<br>BNC2 is a known EOC susceptibility gene. Future studies should further explore the role of DNA methylation in BNC2 [44]. |
| ENSG00000112499 | SLC22A2 | Solute carrier family 22 | Downregulated in pancreatic cancer [45]. High levels of OCT2 (SLC22A2) indicate severe invasion, but also better prognosis in metastatic colorectal cancer (mCRC) patients treated with oxaliplatin-based chemotherapy, possibly because of its role in oxaliplatin susceptibility [46]. |
| ENSG00000135678 | CPM | Carboxypeptidase M | Carboxypeptidase M is not expressed in human renal cell carcinoma tumor cells [47]. |
| ENSG00000184979 | USP18 | Ubiquitin specific peptidase 18 | Decreased expression of USP18 is a reliable prognostic marker for cancer specific survival in muscle invasive bladder cancer (MIBC) [48]. |

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| ENSG00000169398 | PTK2 | Protein tyrosine kinase 2 | Is expressed in several human malignancies (Sulzmaier et al., 2014; Zhao et al., 2009), as well as cervical cancer [49]. |
| ENSG00000141570 | CBX8 | Chromobox homolog 8 | Is a novel oncogene that promotes the proliferation of tumor cells and raises the resistance of neoplasms to chemotherapy in esophageal carcinoma [50]. |
| ENSG00000065361 | ERBB3 | Erb-b2 receptor tyrosine kinase 3 | ROS inducing ERBB3 expression in OVCAR-3 cells [51]. |
| ENSG00000124782 | RREB1 | Ras responsive element binding protein 1 | Is overexpressed in colorectal adenocarcinoma tumors and cell lines [52], and prostate cancer [53]. |
| ENSG00000006634 | DBF4 | Protein DBF4 homolog (Activator of S phase Kinase) | Highly expressed in many cancer cell lines [54]. |
| ENSG00000157764 | BRAF | B-Raf proto-oncogene, serine/threonine kinase | Mutations in BRAF is a frequent event in colorectal cancers (CRC) and BRAF mutations are associated with methylator phenotype in CRC [55-57]. Overexpressed in breast brain metastases [58]. |
| ENSG00000197694 | SPTAN1 | Spectrin, alpha, non-erythrocytic 1 | Linked with tumor progression and ovarian malignancy [59]. |
| ENSG00000135823 | STX6 | Syntaxin 6 | Overexpressed in human cancer as well as, breast, colon, pancreatic, prostate, bladder, skin, testicular, tongue, cervical, liver, lung and gastric cancer and has a role in cellular migration [60]. |
| ENSG00000198682 | PAPSS2 | 3,-phosphoadenosine 5,-phosphosulfate synthase 2 | Expressed in ER-positive breast cancer tissues [61]. |
| ENSG00000146242 | TPBG | Trophoblast glycoprotein | Expressed in colorectal carcinoma [62], bladder, breast, cervix, endometrium, lung, esophagus, ovary, pancreas, stomach carcinomas [63]. |
| ENSG00000125755 | SYMPK | Symplekin | Expressed in human colorectal cancer and promotes tumorigenesis [64]. |
| ENSG00000118898 | PPL | Periplakin | Is highly expressed in triple-negative breast cancer (TNBC) [65]. |
| ENSG00000165030 | NFIL3 | Nuclear factor, interleukin 3 regulated | Highly expressed in basal-like breast cancer and glioblastoma multiforme and NFIL3 expression is strongly correlated with poor prognosis in breast cancer [66]. |
| ENSG00000112473 | SLC39A7 | Solute carrier family 39 (zinc transporter), member 7 | MCF7 cell models of acquired tamoxifen resistance (TamR cells) have increased levels of zinc and zinc transporter, resulting in an enhanced response to exogenous zinc, leading to increased growth and invasion [67]. |
| ENSG00000064042 | NP_055803.1 (LIMCH1) | LIM and calponin homology domains 1 | Overexpressed in ERα-positive breast tumors with PIK3CA mutations [68]. |
| ENSG00000082996 | RNF13 | Ring finger protein 13 | RNF13 gene expression is associated with cancer development [69]. Furthermore, RINF13 is overexpressed in pancreatic cancer [70]. |
| ENSG00000101182 | PSMA7 | Proteasome (prosome, macropain) subunit, alpha type, 7 | Reduced expression in prostate cancer [71]. PSMA7 inhibits the proliferation, tumorigenicity and invasion of A549 human lung adenocarcinoma cells in vitro [72], and PSMA is highly expressed in colorectal cancer cell lines [73]. |
| ENSG00000151240 | DIP2C | Disco-interacting protein 2 homolog C | Somatic mutations in the DIP2C gene have an impact on protein function in breast cancer [74]. |
| ENSG00000136986 | DERL1 | Derlin 1 | Overexpressed in breast-brain metastases [75]. |
| ENSG00000107077 | JMJD2C | Lysine (K)-specific demethylase 4C | Overexpressed in colon cancer cell lines and confers a pro-growth effect on colon cancer cells [76]. |

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| ENSG00000173264 | GPR137 | G protein-coupled receptor 137 | GPR137 is highly expressed in multiple human gastric cancer cell lines; however, Its role in human disease onset has remained to be elucidated [77]. |
| ENSG00000173890 | GPR160 | G protein-coupled receptor 160 | G protein-coupled receptor 160 (GPR160) has been proposed as an oncogene involved in nasopharyngeal carcinoma [78]. |
| ENSG00000070886 | EPHA8 | EPH receptor A8 | mRNA expression in colon cancer [79]. |
| ENSG00000136807 | CDK9 | Cyclin-dependent kinase 9 | Is required for the proliferation of HCC cell lines [80]; furthermore, CDK9 is important for cancer cell survival [81]. |
| ENSG00000150630 | VEGFC | Vascular endhothelial growth factor C | Overexpression of VEGFC in breast cancer cells promotes metastasis to lymph nodes and lungs [82]; furthermore expression of VEGFC has been reported in various types of cancer such as breast, lung, squamous cell, sarcomas, melanomas [83], mesothelioma [84]; gastric [85] and other. |
| ENSG00000138685 | FGF2 | Fibroblast growth factor 2 | Plays an important role in prostate cancer [86], lung [87], and head and neck [88]. |
| ENSG00000158711 | ELK4 | ETS-like transcription factor 4 (ELK4) | Expressed in prostate cancer and contributes to cellular growth [89, 90]. |
| ENSG00000168438 | CDC40 | Cell division cycle 40 | Upregulated in the primary CRC tissues, and promotes CRC cell growth [91]. |
| ENSG00000117298 | ECE1 | Endothelin converting enzyme 1 | Expressed in human prostate cancer cell lines [92], and ovarian carcinoma cells [93]. ECE-1 contributes to invasion and migration in cancer [94, 95]. |
| ENSG00000105647 | PIK3R2 | Phosphoinositide-3-kinase, regulatory subunit 2 (beta) | PIK3R2 mutations have been reported in endometrial tumors and PIK3R2 is considered a novel endometrial cancer gene [96]. |
| ENSG00000127914 | AKAP9 | A kinase (PRKA) anchor protein 9 | The AKAP9 M463I T allele is associated with an increased breast cancer risk in familial breast cancer [97]. |
| ENSG00000118733 | OLFM3 | Olfactomedin 3 | Plays an important role in anoikis resistance, and OLFM3 is expressed in lung, breast and resistant nasal cancer cell lines anoikis [97]. |
| ENSG00000129515 | SNX6 | Sorting nexin 6 | Sorting nexin 6 (SNX6) interacts with breast cancer metastasis suppressor 1 (BRMS 1) protein and favoring transcriptional repression, furthermore, BRMS1-SNX6-HDAC complex may modulate the transcriptional repression [98]. |
| ENSG00000109182 | NP_079363.1 (CWH43) | Cell wall biogenesis 43 C-terminal homolog | Cell Wall Biogenesis 43 C-Terminal Homolog (CWH43) is downregulated in colorectal tumor tissues, but its role in colorectal cancer has not been reported [99]. |
| ENSG00000185250 | PPIL6 | Peptidylprolyl isomerase (cyclophilin)-like 6 | Is a novel gene identified in genomic aberrations associated with prostate cancer progression, but its function has not been characterized [100]. |
| ENSG00000175054 | ATR | ATR serine/threonine kinase | Human colorectal cancer cells require Ataxia telangiectasia mutated and Rad3-related (ATR) for cell cycle progression after IR treatment [101]; ATR is a therapeutic target in cancer [102, 103]; ATR mutations in endometrial cancer are associated with reduced overall survival and disease-free survival [104]. |
| ENSG00000075388 | FGF4 | Fibroblast growth factor 4 | Exogenous FGF4 provides an advantage in cell growth and tumorigenicity of HBL100 and MCF7 breast cancer cells and the cells that expressed FGF4 show an aggressive phenotype, actually, spontaneous metastasis [105-108]. |

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| ENSG00000125304 | TM9SF2 | Transmembrane 9 superfamily member 2 | Expressed in breast cancer cells, and it is propose as a diagnostic biomarker [109]; the expression of TM9SF2 in colorectal cancer (CRC) patients has been associated with poor survival [110]. |
| ENSG00000072274 | TFRC | Transferrin receptor | Expressed in human pancreatic cancer and in neuroendocrine carcinoma of pancreas and it has been proposed as a marker of malignant transformation [111]; furthermore, TFRC is expressed in esophageal squamous cell carcinoma (ESCC), and it can be a prognostic factor in patients with ESCC [112]. TFRC is upregulated in invasive cervical cancer and it is associated with invasion in this type of cancer [113]. |
| ENSG00000141642 | ELAC1 | ElaC ribonuclease Z 1 | Downregulated in colorectal liver metastases [114]. |
| ENSG00000072042 | RDH11 | Retinol dehydrogenase 11 (all-trans/9-cis/11-cis) | Retinol dehydrogenase 11 (RDH11 or PSDR1) is overexpressed in prostate cancer and it has been suggested that it may play role in prostate carcinoma [115, 116]. |
| ENSG00000180667 | YOD1 | YOD1 deubiquitinase | YOD1 was identified as a target of miR-373 in cervical cancer, however, the role of YOD1 in cancer has not yet been elucidate [117]. |
| ENSG00000173253 | DMRT2 | Doublesex and mab-3 related transcription factor 2 | DMRT2 is a transcription factor that is downregulated in clear cell renal cell carcinoma (ccRCC) [118]. |
| ENSG00000101856 | PGRMC1 | Progesterone receptor membrane component 1 | Plays a role in cell growth, cell viability and chemoresistance in endometrial tumors, ovarian cancer, and uterine sarcoma [119-121]; furthermore, this gene is associated with tumorigenesis in lung cancer [122]. |
| ENSG00000141985 | SH3GL1 | SH3-domain GRB2-like 1 | Expressed in human medulloblastoma (MB) cell lines and is a target of miR-128 [123]. |
| ENSG00000173141 | MRP63 | Mitochondrial ribosomal protein L57 | Downregulated in glioma cell lines with 13q deletion [124]. |
| ENSG00000138709 | LARP2 | The ribonucleoprotein domain family, member 1B | Expressed in meningiomas [125]. |
| ENSG00000177189 | RPS6KA3 | Ribosomal protein S6 kinase, 90kDa, polypeptide 3 | RPS6KA3 is frequently mutated in hepatocellular carcinoma (HCC) [126]. |
| ENSG00000164270 | HTR4 | 5-hydroxytryptamine (serotonin) receptor 4, G protein-coupled | Overexpressed in high grade tumours and DU145 and LNCap prostate cancer [127]. |
| ENSG00000122679 | RAMP3 | Receptor (G protein-coupled) activity modifying protein 3 | Expressed in prostate cancer tissue and might be involved in tumor cell growth [128]. |
| ENSG00000186017 | ZNF566 | Zinc finger protein 566 | Zinc finger proteins (ZNF) are implicated in the development of various types of cancer [129-134]. |
| ENSG00000198522 | ZNF512 | Zinc finger protein 512 | |
| ENSG00000171467 | ZNF318 | Zinc finger protein 318 | |
| ENSG00000135502 | SLC26A10 | Solute carrier family 26, member 10 | The solute carriers (SLC) transporters expressed in cancer cells promoting cell growth and SLC members are associated with cancer therapy [135, 136]. |
| ENSG00000075415 | SLC25A3 | Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3 | |
| ENSG00000163848 | SLC12A8 | Solute carrier family 12, member 8 | |
| **Gene-ID** | **Gene symbol** | **Name gene** | **Cancer information not available** |

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| ENSG00000100767 | PAPLN | Papillin, proteoglycan-like sulfated glycoprotein | |
| ENSG00000138032 | PPM1B | Protein phosphatase, Mg2+/Mn2+ dependent, 1B | |
| ENSG00000141198 | TOM1L1 | Target of myb1 like 1 membrane trafficking protein | |
| ENSG00000125534 | C20orf149 | Pancreatic progenitor cell differentiation and proliferation factor | |
| ENSG00000022277 | C20orf43 | Replication termination factor 2 domain containing 1 | |
| ENSG00000121931 | C1orf103 | Ligand dependent nuclear receptor interacting factor 1 | |
| ENSG00000120685 | C13orf23 | Proline and serine rich 1 | |
| ENSG00000103254 | C16orf24 | Family with sequence similarity 173, member A | |
| ENSG00000001460 | C1orf201 | Sperm-tail PG-rich repeat containing 1 | |
| ENSG00000175707 | C1orf172 | Keratinocyte differentiation factor 1 | |
| ENSG00000168175 | C14orf32 | Mitogen-activated protein kinase 1 interacting protein 1-like | |
| ENSG00000166262 | C15orf33 | Family with sequence similarity 227, member B | |
| ENSG00000185567 | Q5BKX7_HUMAN (C14orf78) | AHNAK nucleoprotein 2 | |
| ENSG00000100625 | SIX4 | SIX homeobox 4 | |
| ENSG00000111725 | PRKAB1 | Protein kinase, AMP-activated, beta 1 non-catalytic subunit | |
| ENSG00000166965 | RCCD1 | RCC1 domain containing 1 | |
| ENSG00000153951 | OR4D2 | Olfactory receptor, family 4, subfamily D, member 2 | |
| ENSG00000164366 | CCDC127 | Coiled-coil domain containing 127 | |
| ENSG00000143630 | HCN3 | Hyperpolarization activated cyclic nucleotide gated potassium channel 3 | |
| ENSG00000023909 | GCLM | Glutamate-cysteine ligase, modifier subunit | |
| ENSG00000087470 | DNM1L | Dynamin 1-like | |
| ENSG00000162188 | GNG3 | Guanine nucleotide binding protein (G protein), gamma 3 | |
| ENSG00000168268 | NT5DC2 | 5,-nucleotidase domain containing 2 | |
| ENSG00000167700 | MFSD3 | Major facilitator superfamily domain containing 3 | |
| ENSG00000183340 | JRKL | JRK-like | |

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| ENSG00000174740 | PABPC5 | Poly (A) binding protein, cytoplasmatic 5 | |
| ENSG00000052723 | NP_079349.1 (SIKE1) | Suppressor of IKBKE 1 | |
| ENSG00000054116 | TRAPPC3 | Trafficking protein particle complex 3 | |
| ENSG00000138073 | PREB | Prolactin regulatory element binding | |
| ENSG00000171763 | SPATA5L1 | Spermatogenesis associated 5-like 1 | |
| ENSG00000112972 | HMGCS1 | 3-hydroxy-3-methylglutaryl-CoA synthase 1 | |
| ENSG00000112992 | NNT | Nicotinamide nucleotide transhydrogenase | |
| ENSG00000141994 | DUS3L | Dihydrouridine synthase 3-like | |
| ENSG00000089775 | ZBTB25 | Zinc finger and BTB domain containing 25 | |
| ENSG00000123737 | EXOSC9 | Exosome component 9 | |
| ENSG00000068724 | TTC7A | Tetratricopeptide repeat domain 7A | |
| ENSG00000138363 | ATIC | 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase | |
| ENSG00000159202 | UBE2Z | Ubiquitin-conjugating enzyme E2Z | |
| ENSG00000171861 | RNMTL1 | RNA methyltransferase like 1 | |
| ENSG00000127824 | TUBA1 | Tubulin, alpha 4a | |
| ENSG00000157212 | PAXIP1 | PSX interacting (with transcription-activation domain) protein 1 | |
| ENSG00000084734 | GCKR | Glucokinase (hexokinase 4) regulator | |
| ENSG00000166337 | TAF10 | TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30kDa | |
| ENSG00000149256 | ODZ4 | Teneurin transmembrane protein 4 | |
| ENSG00000004777 | SNX26 | Rho GTPase activating protein 33 | |
| ENSG00000113811 | SELK_HUMAN | Selenoprotein K | |
| ENSG00000165678 | GHITM | Growth hormone inducible transmembrane protein | |
| ENSG00000176261 | ZBTB8OS | Zinc finger and BTB domain containing 8 opposite strand | |
| ENSG00000163964 | PIGX | Phosphatidylinositol glycan anchor biosynthesis, class X | |
| ENSG00000138617 | PARP16 | Poly (ADP-ribose) polymerase family, member 16 | |
| ENSG00000066583 | ISOC1 | Isochorismatase domain containing 1 | |
| ENSG00000179562 | GCC1 | GRIP and coiled-coil domain containing 1 | |
| ENSG00000197568 | HHLA3 | HERV-H LTR-associating 3 | |

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| ENSG00000132846 | ZBED3 | Zinc finger, BED-type containing 3 | |
| ENSG00000135241 | PNPLA8 | Patatin-like phospholipase domain containing 8 | |
| ENSG00000138439 | ALS2CR13 | family with sequence similarity 117, member B | |
| ENSG00000178636 | Q8N7N2_HUMAN | - | |
| ENSG00000166451 | CENPN | Centromere protein N | |
| ENSG00000130363 | RSHL2 | Radial spoke 3 homolog (Chlamydomonas) | |
| ENSG00000106012 | IQCE | IQ motif containing E | |
| ENSG00000166863 | TAC3 | Tachykinin 3 | |
| ENSG00000157890 | MEGF11 | Multiple EGF-like-domains 11 | |

| **Supplementary Table 2.** Genes selected for RT-qPCR validation and methylation analysis | | | | |
|---|---|---|---|---|
| **Gene-ID** | **Gene symbol** | **Gene name** | **GO (Biological process)** | **Epigenetic evidence and cancer involvement** |
| ENSG00000095002 | MSH2 | MutS homolog 2 | - Biological regulation<br>- Cellular component organization<br>- Metabolic process<br>- Reproduction<br>- Response to stimulus | Methylated in hepatocellular carcinoma [8], and Lynch Syndrome tumors [9]. |
| ENSG00000165671 | NSD1 | Nuclear receptor binding SET domain protein 1 | - Cellular component organization<br>- Cellular process<br>- Metabolic process | Methylated in neuroblastoma and glioma [137]. |
| ENSG00000134215 | VAV3 | VAV3 guanine nucleotide exchange factor | - Cellular process<br>- Biological regulation<br>- Immune system process<br>- Metabolic process<br>- Multicellular organismal process<br>- Response to stimulus | Methylated in breast cancer cell lines [138]. |
| ENSG00000163132 | MSX1 | Msh homeobox 1 | - Metabolic process<br>- Developmental process<br>- Biological regulation | Methylated in leukemia (T-ALL, T-linage leukemia) [4], and testicular cancer [5]. Furthermore, MSX1 is a repressor of cell cycle in human ovarian cancer cells [6]. Downregulated in cervical cancer tissue and cervical cell lines [7]. |
| ENSG00000137962 | ARHGAP29 | Rho GTPase activating protein 29 | - Non-annotated gene | Methylated in mantle cell lymphoma [139] . |
| ENSG00000136114 | THSD1 | Thrombospondin, type I, domain containing 1 | - Non-annotated gene | Methylated and candidate tumor suppressor gene in colon cancer [140]. |

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| ENSG00000070731 | ST6GALNAC2 | ST6 (alpha-N-acetyl-neurminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2 | - Metabolic process | Candidate tumor suppressor gene in breast cancer [141]. |
| ENSG00000154556 | SORBS2 | Sorbin and SH3 domain containing 2 | - Metabolic process | Putative tumor suppressor gene involved in cervical carcinogenesis [142]. |
| ENSG0000017326 | GPR137 | G protein-coupled receptor 137 | - Non-annotated gene | Highly expressed in multiple human gastric cancer cell lines; however, Its role in human disease has remained to be elucidated [77]. |
| ENSG00000001460 | C1ORF201 | Sperm-tail PG-rich repeat containing 1 | - Non-annotated gene | No studies have report its relationship with human cancer but plays a role in apoptosis [143]. |

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# DISCUSIÓN

El silenciamiento transcripcional de genes supresores de tumor por metilación anormal de su promotor es un evento epigenético común en cáncer (Esteller, 2007). Hay evidencias que demuestran que DNMT3B tiene un papel importante en el inicio y progresión del cáncer (Beaulieu et al., 2002, Linhart et al., 2007). Además, la relación de la expresión de DNMT3B con la metilación de genes en cáncer se ha demostrado (Ibrahim et al., 2011, Nosho et al., 2009, Roll et al., 2008, Teneng et al., 2015). Aproximadamente, en el 60% de los promotores de genes humanos, hay al menos una isla CpG, lo que sugiere que DNMT3B puede silenciar la expresión de alguno de ellos vía metilación. Hasta ahora se han identificar 5 genes supresores de tumor como blancos directos para su regulación transcripcional por DNMT3B (Fan et al., 2012, Ghoshal et al., 2010, Linhart et al., 2007, Teneng et al., 2015).

De manera general, el objetivo de este trabajo fue identificar genes relacionados con el cáncer que fueran regulados de manera negativa por DNMT3B. Previamente se reportó que la disminución de DNMT3B con un RNA interferente (RNAi) en una línea celular de carcinoma hapatocelular induce la reexpresión de varios genes relacionados con la formación y desarrollo de tumor, reforzando la idea de que DNMT3B funciona como un regulador negativo para algunos genes supresores de tumor (Xu et al., 2005). En este trabajo, la sobre-expresión de DNMT3B en células HaCaT, resultó en la disminución de la expresión de 1085 genes, de los cuales151 genes presentan una isla CpG en su promotor. Esto sugiere que la disminución de la expresión de los genes se debió a la metilación de su promotor por la sobre-expresión de DNMT3B. Este resultado es similar con lo reportado por Xu *et al*. (2005), donde reportan la reexpresión de algunos genes con isla CpG en su promotor después del knockdown de DNMT3B en células de cáncer hepatocelular. En este mismo sentido, se ha reportado que DNMT3B muestra preferencia para la metilación de regiones densas en CpG y es excluida de promotores activos (Baubec et al., 2015). Además, la represión por metilación requiere promotores con un alto contenido de citosinas metiladas (Weber et al., 2007).

DNMT3B participa en el inicio y progresión de cáncer, favoreciendo la metilación de genes supresores de tumor que participan en vías importantes que se ven afectadas en cáncer (Esteller, 2007, Soejima et al., 2003). Un escenario similar puede ser visto en este trabajo, los 151 genes identificados, participan en procesos de comunicación celular, procesos biológicos y procesos metabólicos que se ven afectados durante la carcinogénesis. Además, identificamos 5 genes considerados supresores de tumor, 22 genes asociados al silenciamiento transcripcional por metilación en varios tipos de cáncer humano, y al menos 61 genes involucrados en la carcinogenésis (ver tabla 1S del capítulo 1).

La selección de un grupo de genes para la validación de los datos de expresión global por RT-qPCR, demostró que la expresión del RNAm de los genes VAV3, GPR137 y SORBS2 disminuyó de manera significativa luego de la sobre-expresión de DNMT3B en células HaCaT. Los genes MSH2, C1ORF201, ST6GALNAC2 y MSX1, también disminuyeron la expresión de su RNAm, aunque el resultado no fue significativo. La disminución o la pérdida de su expresión total por metilación del gen MSH2 se ha reportado en cáncer colorectal (Lawes et al., 2005), y cáncer de pulmón de células no pequeñas (Lahtz and Pfeifer, 2011, Wang et al., 2003). La disminución de la expresión del gen ST6GALNAC2 se ha observado en pacientes con cáncer de mama y se ha propuesto como un supresor de metástasis (Murugaesu et al., 2014). La disminución de la expresión de los genes MSX1 y SORBS2 se ha reportado en cáncer cervical, y se han propuesto como genes supresores de tumor en este tipo de cáncer (Backsch et al., 2011, Shim et al., 1998). Estos resultados sugieren que, los genes VAV3, GPR137 y SORBS2 son genes regulados negativamente por DNMT3B, además permite indicar la importancia de DMNT3B en la regulación de genes relacionados con el cáncer.

Considerando los resultados de validación y el hecho de que los genes VAV3, GPR137 y SORBS2 son genes que participan en procesos celulares que se ven afectados en cáncer, como es, control del crecimiento celular, proliferación y migración celular, consideramos a estos genes para analizar si la disminución de su expresión pudo deberse a la metilación de su promotor. Los resultados del análisis de metilación, mostraron un aumento en la metilación del promotor del gen VAV3. Exactamente, 12 sitios CpG cercanos al sitio de inicio de la transcripción fueron metilados en el promotor de VAV3. Esto sugiere que VAV3 es regulado vía

metilación. VAV3 es un factor intercambiador de nucleótido de guanina involucrado en la regulación de las GTPasas Rho, y en varios procesos celulares, como es, la organización del citoesqueleto, regulación de la expresión génica, transformación celular y oncogenésis (Bustelo, 2000, Uen et al., 2015). Además, nuestros datos son consistentes con lo reportado en la literatura. Recientemente, se ha estudiado el estado de metilación del gen VAV3 en cáncer. En este sentido, la metilación de VAV3 se ha reportado en líneas celulares de cáncer de mama (Loss et al., 2010), y en muestras de paciente de cáncer gástrico, donde VAV3 se ha considerado como un marcador de metilación para estimar la fracción de células cancerosas en muestras de cáncer gástrico (Zong et al., 2016). Se ha propuesto además, que la metilación del gen VAV3 en muestras de suero de pacientes con cáncer gástrico, ofrece una alternativa de detección de este tipo de cáncer por un método no invasiva (Li et al., 2016). De acuerdo a los resultados de este trabajo, resultó sorprendente, haber encontrado regiones metiladas de la isla CpG de VAV3 en las células HaCaT donde no se sobre-expreso a DNMT3B, sin embargo la metilación de este gen en células normales también se ha reportado (Li et al., 2016, Zong et al., 2016). Poco se sabe de la regulación de la expresión de VAV3, sin embargo, nuestros datos y los ya reportados, parecen indicar que la regulación de la expresión de VAV3 puede ser por metilación de su promotor. Otro dato que apoya nuestros resultados, es que, mediante experimentos con RNAi, la disminución de la expresión de DNMT3B en la línea celular de hepatocarcinoma SSMC-7721, resultó en la reexpresión de 115 genes, dentro de los cuales se encontró VAV3 (Xu et al., 2005). Por tanto, los resultados de este estudio y los datos previos, podrían indicar que VAV3 puede ser un gen regulado por DNMT3B vía metilación. Hasta ahora, no hay reportes del efecto de la disminución de la expresión de VAV3 por metilación en cáncer, por lo que se sugiere investigar su papel en la carcinogénesis.

La formación de complejos DNMT3B con factores de la transcripción puede favorecer la metilación de promotores de genes (Hervouet et al., 2009), y se sabe que algunos factores de la transcripción no pueden unirse al promotor cuando el CG dentro de su secuencia de unión está metilado (Tate and Bird, 1993). Un análisis *in silico* usando el programa CONSITE, para predecir sitios de unión para factores de la transcripción dentro de la región donde se encontraron cambios en la metilación de los 12 CpGs del promotor de VAV3, apuntó que hay un sitio de

unión para el factor de la transcripción Sp1, AP2-alpha, MZF, Hen-1, Thing1-E4 y E2F. Es bien conocido que la metilación del CpG dentro del sitio de unión para Sp1 generalmente interfiere con su unión y puede afectar la transcripción (Cao et al., 2000, Kim et al., 2016). El factor de la transcripción E2F no se puede unir a su sitio de unión cuando está metilado (Tate and Bird, 1993, Campanero et al., 2000). Para algunos promotores, AP2-alpha puede actuar como un supresor para la unión de Sp1, también la unión de AP2-alpha al DNA puede iniciar el silenciamiento transcripcional por DNMTs (Bennett et al., 2009, Liu et al., 2007). Es posible que la metilación de los sitios de unión para Sp1, AP2-alpha y E2F localizados en la región densamente metilada del promotor de VAV3 pueda inhibir su unión y resultar en la inactivación transcripcional del gen. Este evento puede explicar la disminución de la expresión de VAV3 en células HaCaT con sobre-expresión de DNMT3B. Además, el factor de la transcripción Sp1 y algunos factores E2F, se sabe que pueden unirse a DNMT3B (Hervouet et al., 2009). Resulta importante valorar la unión de dichos factores de la transcripción con DNMT3B en el promotor de VAV3.

Adicionalmente, en este trabajo, se valoró la expresión del RNAm de VAV3 en diferentes líneas celulares de cáncer. Los resultados muestran diferencias en la expresión de dicho gen en las diferentes líneas celulares analizadas. La expresión del RNAm de VAV3, puede estar aumentada o disminuida dependiendo del contexto celular. Mientras que su expresión es elevada en cáncer de próstata, donde su función es de oncogén (Dong et al., 2006), su expresión está disminuida en cáncer gástrico (Zong et al., 2016). En este trabajo encontramos que las línea celular de cáncer cervical C-33A, la línea celular de cáncer de pulmón de células no pequeñas A549 y de adenocarcinoma de mama MCF-7, muestran disminución de la expresión del gen VAV3. Esto podría sugerir un papel importante de VAV3 en cáncer, sugiriendo su participación en la formación y crecimiento del tumor, apoptosis, metástasis, y angiogénesis (Tan et al., 2014a, Tan et al., 2016, Tan et al., 2014b). Además, también se valoró la expresión de DNMT3B en muestras de pacientes con cáncer cervical y en las diferentes líneas celulares de cáncer. Los resultados de expresión mostraron, un aumento de la expresión de DNMT3B en muestras de cáncer, comparada con tejido normal, y con la línea celular no tumoral HaCaT. De acuerdo a los resultados obtenidos en este trabajo, se puede especular que la disminución de VAV3 en las líneas C-33A, A549 y MCF7 puede deberse a

la metilación de su promotor. Se sugiere valorar el estado de metilación del gen VAV3 en las líneas celulares y en muestras de pacientes con cáncer cervical.

La sobre-expresión de DNMT3B en células HaCaT, resultó en la disminución de la expresión del RNAm de los genes GPR137 y SORBS2, sin embargo la metilación en su promotor no aumentó. SORBS2, es una proteína scaffold involucrada en el ensamble de complejos de señalización de fibras de estrés y actina (Kawabe et al., 1999, Kioka et al., 2002, Wang et al., 1997). La función antitumoral de SORBS2 se ha demostrado (Roignot and Soubeyran, 2009, Taieb et al., 2008). Además, se ha propuesto como un gen supresor de tumor, y la disminución y perdida de su función en cáncer cervical y cáncer de páncreas se ha reportado (Backsch et al., 2011, Taieb et al., 2008). Sin embargo no hay evidencias de la regulación transcripcional de SORBS2 vía metilación. Para determinar si la disminución de la expresión de SORBS2 por la sobre-expresión de DNMT3B se debió a metilación de su promotor, se analizaron 30 sitios CpG cercanos al sitio de inicio de la transcripción, los 30 sitios se encontraron metilados, tanto en células HaCaT que sobre-expresaron a DNMT3B y en las que no. Esto sugiere que la disminución de la expresión de SORBS2 en células HaCaT después de la sobre-expresión de DNMT3B no fue por metilación.

La predicción de factores de la transcripción alrededor de los 30 CpGs, indican un sitio de unión para IRF1. Además, se confirmó en GeneCards-Human/Gene Database (http://www.genecards.org), que el sitio de unión para IRF1 está presente en el promotor de SORBS2. Las proteínas IRF tienen un papel central en la regulación de la expresión de genes (Tamura et al., 2008). Resulta interesante el hecho de que en el análisis de expresión global, el gen IRF1 disminuyó su expresión (Zscore -2.682289), además tiene isla CpG en su promotor. Sugerimos que la disminución de la expresión de SORBS2 pudo ser de manera indirecta, por la disminución de la expresión de IRF1.

Adicionalmente, se valoró el nivel de expresión del RNAm de SORBS2 en líneas celulares de cáncer. Aun cuando se ha reportado la disminución de la expresión de SORBS2 en cáncer cervical y cáncer de páncreas (Backsch et al., 2011, Taieb et al., 2008), no hay reportes de expresión de este gen en sus líneas

celulares. Los resultados en este trabajo mostraron una expresión mayor en células de cáncer comparado con la célula no tumoral HaCaT.

El papel de GPR137 en cáncer se relaciona con la regulación del crecimiento celular, proliferación, invasión y apoptosis (Cui et al., 2015, Ren et al., 2016, Shao et al., 2015, Wang et al., 2015b). Similar a SORBS2, no se encontró aumento en la metilación del promotor de GPR137 y no hay reportes de la regulación transcripcional de este gen por metilación de su promotor. Es probable que la disminución de la expresión de GPR137 y SORBS2 haya ocurrido por algunos eventos o mecanismos adicionales. Por ejemplo, represión transcripcional independiente de metilación por DNMT3B, como se ha reportado para algunos genes (Fan et al., 2012, Haney et al., 2015).

Al igual que se valoró la expresión del RNAm de VAV3 y SORBS2 en diferentes líneas celulares de cáncer, también se midió el nivel de expresión del RNAm de GPR137 en líneas celulares de cáncer. El resultado obtenido mostró expresión diferente entre las líneas celulares. La línea celular de cáncer cervical HeLa y de cáncer de pulmón A549 mostraron un nivel de expresión de GPR137 menor en comparación con las otras líneas celulares de cáncer cervical y cáncer de mama, además de la línea celular no tumoral HaCaT. Debido a que la línea celular HeLa y A549 muestran un nivel de expresión mayor de DNMT3B que la línea no tumoral HaCaT, resulta interesante valor el estado de metilación de GPR137 en ambas líneas celulares.

En conclusión, los resultados obtenidos en este trabajo sugieren que la sobre-expresión de DNMT3B en células HaCaT, regula la expresión de genes relacionados con el cáncer, disminuye la expresión de 151 genes con isla CpG, y disminuye la expresión del gen VAV3 vía metilación de su promotor, sugiriendo que VAV3 puede ser regulado de manera directa por DNMT3B. Estos hallazgos indican la importancia de DNMT3B en la regulación de la expresión de genes y en cáncer humano.

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