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EVALUACIÓN DE ELEMENTOS CELULARES QUE INTERACTÚAN CON LAS PROTEÍNAS E6 DE LOS VPH DE ALTO RIESGO

T E S I S

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CONTENIDO

LISTA DE ABREVIATURAS

ºC	Grados Centígrados
aa	Aminoácido
AP1	Proteína Activadora 1
AR	Alto riesgo
CaCu	Cáncer Cérvico uterino
CDK2	Cinasa dependiente de ciclina 2
CR1/2	Región conservada 1/2
DED	Dominio Efector de Muerte
DNA	Ácido Desoxirribonucleico
E2BS	Sitio de unión a E2
E2F	Factor de Elongación 2
E6AP	Proteína Asociada a E6
FHL-2	Proteína 2 de cuatro y medio dominios LIM
Hrs	Horas
IARC	Asociación Internacional de Investigación en Cáncer
KDa	Kilo Daltones
LCR	Región Larga de Control
ORF	Marco Abierto de Lectura
PAGE	Electroforesis de Gel de Poliacrilamida
PCR	Reacción en Cadena de la Polimerasa
PBM	Motivo de Unión a dominios PDZ
PBS	Buffer Salino de Fosfatos
PKA	Proteína Cinasa A
pRb	Proteína del Retinoblastoma
RNA	Ácido Ribonucleico
SNX	Sorting Nexin
TNF	Factor de Necrosis Tumoral
VPH	Virus del Papiloma Humano
Vps	Vacuolar protein sorting

RESUMEN

El Cáncer cérvico (CaCu) uterino representa un serio problema de salud pública, ocupando la tercera posición en neoplasias asociadas a la mujer en el mundo. Numerosos estudios epidemiológicos y moleculares han reconocido la infección por Virus del Papiloma Humano (VPH) como causa necesaria para el desarrollo de CaCu más no suficiente, es aquí donde otros factores juegan un papel importante en el establecimiento del CaCu. Los VPH deben su carácter oncogénico a la expresión continua de las oncoproteínas E6 y E7.

La expresión de la proteína E6 es importante en el establecimiento de la carcinogénesis viral, dicha proteína es capaz de interactuar con diversos blancos celulares y modular su función. Recientemente Rozeblantt-Rosen *et al.* (2012) demostraron los resultados de un interactoma empleando proteínas de virus oncogénicos. Dentro de los blancos celulares potenciales de interacción con E6 y E6* se encontró a Sorting Nexin 27 (SNX27),Vps26A (*Vacuolar protein sorting*), Vps35, Zixina y FHL-2.
El objetivo de este trabajo fue evaluar nuevos blancos celulares regulados por la proteína E6 en términos de interacción y relevancia biológica con el fin de entender la historia natural de la infección por el VPH y su progresión hacia cáncer.

Los resultados encontrados en este trabajo demuestran la capacidad de las proteínas E6 de los VPH de alto riesgo, así como las formas E6*, de interactuar y modular, tanto las actividades como la distribución celular de las proteínas FHL-2, SNX27 y procaspasa 8, demostrando la amplia gamma de procesos celulares alterados por la presencia de E6 y posicionándola como un elemento clave en la comprensión del proceso carcinogénico inducido por VPH.

ABSTRACT

Cervical cancer is considered a major public health problem in developing countries, occupying the third place in incidence among women worldwide. Persistent infection with the so-called High Risk Human Papilloma Virus has been recognized as a pre-requisite in cervical cancer development along with other host and cellular factors by several epidemiologic and molecular studies. HPV oncogenic potential is due to the constitutive expression of E6 and E7.

The E6 oncoprotein expression plays a role in the carcinogenesis drove by HPV, since it is able to interact and modulate a plethora of cellular proteins and functions. Recently, Rozeblantt-Rosen *et al.* (2012) showed the results of an interactome analysis performed with the oncogenic viral proteins, such as HPV. Among the identified proteins we found Sorting Nexin 27 (SNX27),Vps26A (*Vacuolar protein sorting*), Vps35, Zixina and FHL-2 as potencial interacting partners for E6 and E6*.

The main goal of the project was to evaluate new interacting partners for E6 in terms of biological relevance aimed to understand the natural history of the infection and the carcinogenesis induced by HPV.

The results demonstrated the capacity of E6 oncoproteins to interact with and modulate the activities and cellular distribution of FHL-2, SNX27 and Procaspase 8, showing the wide range of cellular processes altered in the presence of E6 pointing its relevance as a key modulator in cancer development.

INTRODUCCIÓN

GENERALIDADES

El Cáncer es un conjunto de enfermedades caracterizado por el crecimiento descontrolado de las células con la capacidad de invadir tejidos adyacentes u otros órganos mediante un proceso llamado metástasis (Parkin, 2006). El cáncer inicia con una sola célula en un proceso carcinogénico gradual y que requiere de múltiples pasos. De acuerdo a la Organización Mundial de la Salud (OMS) las muertes por cáncer representan la primer causa de muerte a nivel mundial (Globocan 2012) y cerca del 70% de las muertes asociadas a cáncer suceden el países en vías de desarrollo, como lo es México. Los agentes etiológicos del cáncer varían desde mutaciones somáticas, exposición a carcinógenos hasta infecciones por organismos, principalmente bacterias y virus, que son capaces de estimular la entrada a ciclo celular y la subsecuente acumulación de mutaciones ayudando al establecimiento de la enfermedad (*IARC report* Capitulo 100B; 2009).

CÁNCER CÉRVICO UTERINO

Actualmente, el cáncer cérvico uterino (CaCu) representa un serio problema de salud pública siendo la tercera causa de muerte por cáncer en mujeres con 265 653 muertes anuales y ocupando la tercera posición en incidencia de neoplasias a nivel mundial con cerca de 527 604 nuevos casos. En México, cada año, son diagnosticados cerca de 13 960 nuevos casos, de los cuales 4 769 fueron decesos (Globocan 2012). Numerosos estudios epidemiológicos y moleculares han reconocido la infección por Virus del Papiloma Humano (VPH) como causa necesaria para el desarrollo de CaCu más no suficiente, es aquí donde otros factores juegan un papel importante en el establecimiento del CaCu. Entre estos factores encontramos: tabaquismo, uso de anticonceptivos, edad de inicio de vida sexual, multiparidad, que junto con el tipo viral, definen el fenotipo maligno (Bouvard *et al.,* 2008; IARC report, 2009).

La infección por VPH, es una enfermedad de transmisión sexual muy común, más de la mitad de los adultos sexualmente activos cursarán con una infección relacionada a este virus en algún momento de su vida. Esta infección se detecta frecuentemente en mujeres jóvenes (18-30 años) sexualmente activas, sin embargo, en la mayoría de los casos (90%) la infección es transitoria y persiste en solo una pequeña fracción (menos del 5%), quienes poseen una mayor probabilidad de desarrollar cáncer.

La infección por VPH no solo está asociada al desarrollo de CaCu, si no también al desarrollo de cáncer de vulva, vagina, pene, ano y cierto tipo de tumores de cabeza y cuello (Manzo-Merino *et al.,* 2013). A la fecha se han descrito 174 tipos de VPH de los cuales, 18 han sido definidos como agentes causales de cáncer (Bzhalava *et al*., 2013). Los VPH se clasifican como de Bajo Riesgo (VPH-BR) y de Alto Riesgo (VPH-AR), dicha clasificación corresponde al potencial oncogénico de los distintos tipos virales, los primeros se asocian con el desarrollo de verrugas y displasias benignas, mientras que los segundos se asocian al cáncer en distintos sitios anatómicos (Tabla 1). Los VPH exhiben un alto grado de tropismo celular, infectando solo células epiteliales de la capa basal a las cuales llegan mediante micro lesiones en el epitelio (Doorbar, 2005).

Sitio anatómico	**Tipo de VPH**	**Frecuencia atribuible (%)**	**% de todos los cánceres**
Cérvix	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 67, 68, 69, 73, 82	100	4.5
Otros cánceres ano genitales, excluyendo cérvix			
Vulva	16, 18, 33	40	0.2
Vagina	16	50	0.2
Pene	16, 18	50	0.1
Ano	16, 18, 33	80	0.2
Cáncer del tracto Aero digestivo			
Cavidad oral	16, 18	23	0.1
Orofaringe y amígdalas	16	35	0.1

Tabla 1. Tipos de VPH asociados a cáncer, frecuencia atribuible de todos los cáncer de ese sitio anatómico y su frecuencia alrededor del mundo. *IARC report* Capitulo 100B, 2009; Parkin, 2006; Bzhalava *et al*., 2013.

VIRUS DEL PAPILOMA HUMANO

El VPH es un virus pequeño de aproximadamente 55nm, no envuelto que pertenece a la familia *Papillomaviridae* (zur Hausen y de Villiers, 1994). Estos virus están ampliamente distribuidos en la naturaleza e infectan específicamente el epitelio escamoso de más de 20 especies diferentes de mamíferos, así como aves y reptiles (Rector y Rants, 2013). La cápside del VPH está constituida por 72 capsómeros (60 hexámeros y 12 pentámeros), conformada por las proteínas estructurales L1 y L2 que albergan el genoma viral (Buck *et al*., 2013). El genoma viral, consiste en un DNA circular de doble cadena de 7200-8000 pares de bases (pb), que contiene más de 10 marcos de lectura abiertos (ORFs) y generalmente una hebra es transcripcionalmente activa (zur Hausen, 1996). El genoma del virus (Figura 1), para su estudio, ha sido dividido en tres regiones: Región temprana (E: Early) que codifica para los genes implicados en replicación y mantenimiento del genoma viral (E1-E8), Región tardía (L: Late), la cual codifica a las proteínas estructurales de la cápside L1 y L2, y por último, la región larga de control (LCR: Long Control Region), la cual contiene los sitios de regulación de la transcripción y replicación viral (Walboomers *et al.,* 1999).

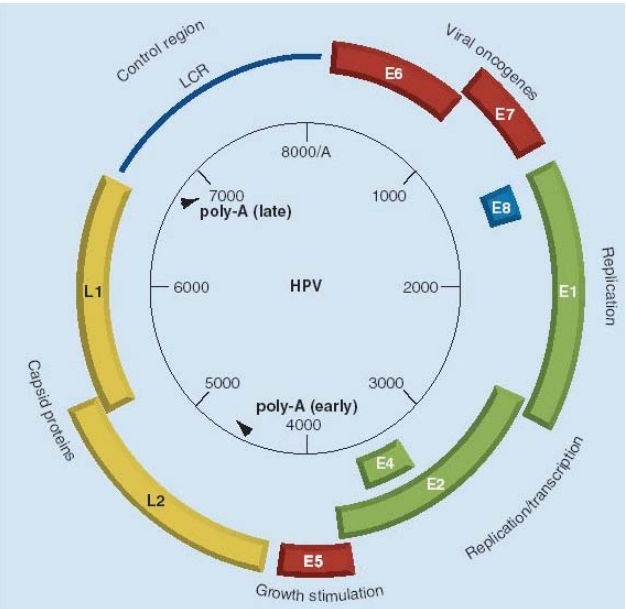


Figura 1. Esquema representativo del genoma del VPH. Se muestran las diferentes proteínas codificadas por el VPH y se especifican los procesos en los cuales están implicadas. En rojo se representan los oncogenes, en verde están representadas las proteínas implicadas en replicación y transcripción del genoma viral y en amarillo se representan las proteínas estructurales. La línea azul corresponde a la región larga de control.

Región Larga de Control

Esta sección del genoma viral se caracteriza por poseer diversos sitios de regulación de la transcripción y replicación viral, no posee marcos abiertos de lectura. Su tamaño varía dependiendo del tipo viral abarcando de un 7 a un 11% del genoma, en el caso de los VPH genitales esta región es de aproximadamente 850 pb (Zheng y Baker, 2006). Factores celulares y virales interactúan con esta región, como las proteínas E1 y E2 que modulan la actividad transcripcional e inician la replicación del virus, algunos componentes de la maquinaria basal de transcripción como SP1 y TBP, receptores de glucocorticoides que modulan positivamente la transcripción así como factores específicos de tejido como KRF, Skn-Ia/i, TEF, AP1, entre otros (Bernard, 2013).

Proteínas L1 y L2

L1 y L2 son las proteínas estructurales de la cápside de los viriones de VPH, L2 es el componente minoritario de la cápside, tiene un peso molecular de 43 a 53 KDa y se produce en células que expresan E4 al igual que L1 y juega un papel primordial en la introducción del genoma viral dentro del núcleo durante el establecimiento de la infección y en el encapsulamiento del mismo durante las etapas tardías de la replicación viral (Buck *et al*., 2013; Wang y Roden, 2013). L1 es la proteína mayoritaria, pesa 57 KDa y que conforma cerca del 80 % de la cápside, se expresa después de L2. La cápside contiene 360 copias de L1 y aproximadamente 12 copias de L2, organizados en 72 capsómeros de una partícula icosahédrica (Trus *et al*., 2005).

Proteína E1

La proteína E1 es la de mayor longitud y la más conservada entre los distintos tipos de VPH, su longitud varía dependiendo del tipo viral y fluctúa entre los 593 y los 681 aminoácidos con un peso molecular de 67.5-76.2 KDa. E1 es una proteína con actividad de helicasa necesaria para la replicación del DNA viral, se divide en tres regiones funcionales: un domino N-terminal, susceptible de fosforilación; une región espaciadora que varía dependiendo el tipo viral y un dominio C-terminal donde radica la actividad de helicasa (Bergvall *et al*., 2013).

E1 se une de manera débil a las secuencias de origen en la LCR, pero esta unión es facilitada por su asociación con E2, los sitios de unión para E2 están cercanos a las secuencias de reconocimiento de E1, esta asociación permite a E1 formar hexámeros con una alta afinidad por el DNA iniciando el proceso de replicación. Estos complejos de E1 desenrollan eficientemente el DNA superhelicoidal con la ayuda de proteínas chaperonas (Huges y Romanos, 1993).

Proteína E2

E2 es una proteína nuclear de 52 KDa que tiene como función la replicación y regulación de la transcripción del genoma viral. Consta de tres dominios, una región N-terminal que contienen el dominio de transactivación (E2TAD) que promueve la oligomerización entre moléculas de E2 unidas al DNA. En el extremo C-terminal E2 posee un dominio de unión a DNA, dichos dominios están conectados por una región bisagra (McBride, 2013). Los dímeros de E2 se unen a secuencias palindrómicas consenso (ACCN6GGT) denominadas sitios de unión de E2 (E2BSs). En la LCR de los VPH-AR hay cuatro E2BSs localizados hacia el 5´ del promotor viral que regula la expresión de los genes tempranos (Rashmi y Hegde, 2002).

La unión de E2 al ADN provoca la represión o activación de dicho promotor y la replicación del ADN viral. A su vez, estas funciones dependen de la concentración intracelular de E2. A bajas concentraciones, E2 activa la expresión de genes tempranos, mientras que a concentraciones elevadas la reprime, ya que interfiere con la unión de factores de transcripción tales como TFIID y Sp1 al bloquear sus secuencias de reconocimiento, las cuales se superponen con las de E2BS. Esta regulación de la expresión viral contribuye al control del número de copias en células indiferenciadas. Durante la diferenciación, hay un *switch* en el promotor tardío, el cual, no es reprimido por E2, lo que resulta en un aumento en la expresión de E1 y E2 dando como resultado la amplificación del ADN viral (McBride y Myers, 1997).

Además de su papel como regulador transcripcional, E2 tiene una función antiproliferativa, ya que puede reprimir el crecimiento celular e inducir apoptosis, en

parte mediante la represión de la trascripción de E6 y E7, así como por el consecuente aumento de p53. Además, E2 es capaz de inducir arresto en G1 por medio de la activación del complejo p21/WAF, inhibidor del complejo ciclina E/CDK2 (McBride, 2013).

Proteína E4

La secuencia codificante de E4 está contenida dentro del ORF de E2, aunque se encuentra ubicada en la región temprana, esta se expresa como un gen tardío al encontrarse regulado por un promotor específico de diferenciación. La proteína se sintetiza a partir del procesamiento del RNA mensajero E1^E4, la secuencia resultante de aproximadamente 260 pb codifica una proteína con un peso aproximado de 10 KDa. Los niveles de de expresión de E4 son elevados durante las etapas tardías del ciclo viral por lo que se acumula en células diferenciadas de las capas epiteliales superiores localizándose en filamentos intermedios de queratina del citoplasma (Raj *et al*., 2004).

E4 causa el colapso de dichas queratinas y esto se ha relacionado con la liberación de los viriones. Por otro lado se ha demostrado que E1^E4 de VPH 16 causa arresto en la fase G2 del ciclo celular cuando se expresa en células HeLa y SiHa sugiriendo un papel antagónico a la proliferación celular inducida por E7 durante la etapa productiva de la infección, así como el requerimiento de E1^E4 y E2 para inhibir la división celular durante el ciclo viral (Clare *et al*., 2002).

Proteína E5

E5 es una proteína de 83 aa con un peso molecular de 14 KDa con un potencial transformante débil, se localiza preferentemente en retículo endoplásmico, aparato de Golgi y en la membrana plasmática (DiMaio y Petti, 2013).

Diversos estudios han demostrado que E5 de VPH16 es capaz de incrementar la eficiencia de inmortalización de queratinocitos primarios inducida por E6 y E7, incrementar la motilidad y capacidad de invasión mediado por su dominio N-terminal (Stöppler *et al.,* 1996; Barbaresi *et al*., 2010; Lewis *et al.,* 2008).

Proteína E8^E2

La proteína E2 es el producto de la fusión del ORF de E8 con una parte de la proteína E2. Dicha proteína de fusión de 20 KDa, es capaz de reprimir la replicación y transcripción viral, y se piensa que puede jugar un papel en el mantenimiento de latencia viral observada en las células basales del epitelio infectado (Lace *et al*., 2012).

Las proteínas E8^E2 ejercen efectos transcripcionales y de replicación al interactuar con sitios cis definidos interfiriendo con la activación transcripcional dependiente de E2, a través una competición por los sitios de unión a E2 (Lace *et al*., 2008).

Proteína E7

La proteína E7 se compone de aproximadamente 100 aa y está codificada dentro de la región temprana del genoma viral, tiene un peso de 10 KDa y se traduce como parte de un mensajero bicistrónico junto con el ORF de E6 en el caso de los VPH de alto riesgo (Liu *et al*., 2007). Esta proteína posee la mayor capacidad transformante mediante la unión a proteínas celulares supresoras de tumores de la familia pRb, que controlan la replicación celular (Seedman *et al*., 1991).

E7 posee un dominio de unión a zinc en la región C-terminal, que se utiliza para su dimerización o multimerización, y un sitio de fosforilación en su región N-terminal. E7 contiene dos secuencias conservadas denominadas CR1 y CR2 que contribuyen a la actividad transformante de las oncoproteínas E7 de los VPH de alto riesgo. Un motivo Leu-X-Cys-X-Glu (LXCXE) ubicado en CR2 es necesario y suficiente para la asociación de E7 con la proteína supresora de tumores del retinoblastoma pRb, así como a las proteínas *pocket*, p107 y p130, pertenecientes a la familia RB (McLaughlin-Durbin y Münger, 2009).La proteína E7 del los papilomavirus de alto riesgo se asocian preferencialmente con la proteína pRb unida a E2F, marcándola para su degradación por medio de la vía proteosomal. Esta desestabilización de pRb/E2F, promueve un descontrol celular en el mecanismo de salida de G1 y del ingreso a la fase S (Liu *et al*., 2006).

Las proteínas E7 de los VPH de alto riesgo pueden interactuar con $p21^{CIP1}$ y $p27^{KIP1}$, inhibidores de los complejos ciclina-cinasa, lo que resulta en la elevación de los niveles de las ciclinas E y A. La interacción de E7 con estas proteínas, así como con las desacetilasas de histonas y AP1, inducen múltiples respuestas celulares, incluyendo la estabilización de p53, quien contrarrestaría esta replicación celular anormalmente estimulada mediante el incremento de la apoptosis. Sin embargo, la proteína E6 degrada a p53 y por tanto bloquea esta respuesta celular (Roman y Munger, 2013).

Proteína E6

E6 es una proteína que se expresa tempranamente durante una infección por VPH. La proteína E6 consta de aproximadamente 150 aminoácidos con un peso molecular de 16 a 18 KDa dependiendo del tipo viral (Figura 2). E6 posee cuatro motivos CX_2C-X_{29}-CX_2C que permiten la formación de dos dedos de zinc, lo cuales, están conectados por un interdominio de 36 aminoácidos y flanqueados por un dominio hidrofílico amino terminal, y por un dominio carboxilo terminal corto, que en el caso de los VPH de alto riesgo, contiene un motivo de unión a dominios PDZ (PBM), que se sobrelapa con un sitio proteína cinasa A (PKA). Los dominios dedos de zinc se contraponen entre sí de manera simétrica, formando una estructura seudodimérica, que junto con el interdominio, crean una hélice que los hace rígidos (Howie *et al*., 2009).

Los RNA mensajeros tempranos de los VPH de alto riesgo son bicistrónicos, mientras que los VPH de bajo riesgo son monocistrónicos; los RNA mensajeros E6/E7 de los VPH-AR sufren un proceso de corte y empalme (*splicing*) durante el cual, se generan transcritos pequeños y consecuentemente una gran parte de la proteína E6 completa puede dejar de producirse (Smootkin *et al.,* 1986). Entre los polipéptidos que se generan a consecuencia del *splicing* se encuentran cuatro productos del gen E6, llamados E6* I-IV para el VPH-16, mientras que para el VPH-18 únicamente se ha descrito una forma E6*I (Roggenbuck *et al*., 1991). Una característica importante de los productos potenciales E6* (Figura 2), es que estos pierden la secuencia carboxilo terminal X-T/S-X-V/L, la cual, es utilizada por la proteína E6 completa para su interacción con proteínas que contienen dominios PDZ (Pim *et al*., 2012).

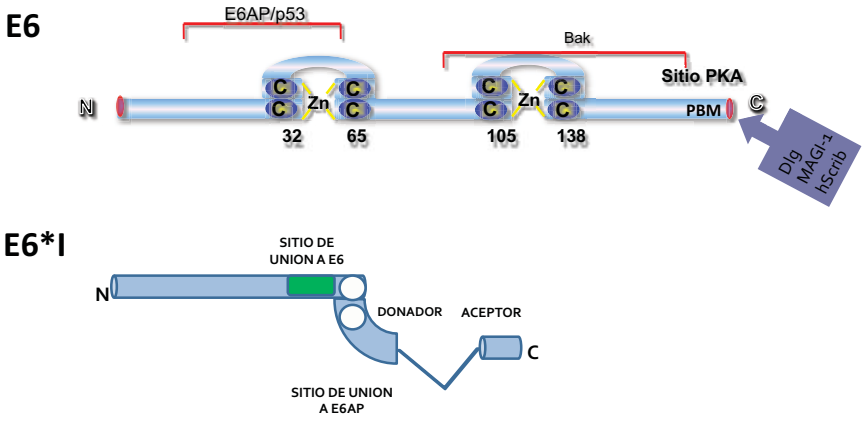


Figura 2. Imagen representativa de las proteínas E6 y E6*I de VPH-18. E6 es una proteína que sufre un proceso de corte y empalme generando formas truncas llamadas E6* que carecen de la Proción C-terminal de la proteína. La imagen muestra los sitios más importantes de interacción con otras proteínas y el motivo de unión a proteínas con dominios PDZ; así como los sitios donadores y aceptores que participan en el proceso de *splicing*. E6*I pierde el PBM y posee 12 aa únicos en la porción C-terminal de la proteína debido al cambio en el marco de lectura producto del *splicing*.

Los productos E6*I se detectan de manera frecuente en tumores asociados a virus de alto riesgo, por lo cual, se creía que estas isoformas de E6 poseían capacidades transformantes. Sin embargo, los hallazgos mostraron que la co-expresión de E6*I con E6 y E7 en queratinocitos humanos produce menos inmortalización de colonias celulares que las obtenidas en ausencia de E6*I (Huibregtse y Beaudenon, 1996). Posteriormente, se observó que el producto E6*IV generado a partir del VPH 16, era capaz de inhibir la degradación de p53 mediada por E6 *in vitro e in vivo* (Shally *et al*., 1996). Por otro lado, Pim y Banks (1999) demostraron que la expresión ectópica de la proteína E6*I de VPH18 ejercía un efecto antiproliferativo en células de tumores de cérvix positivas a VPH. Este efecto lo atribuyeron a la capacidad de E6*I de inhibir la degradación que la proteína E6 completa ejerce sobre p53, así como al incremento de transactivación transcripcional por p53. Observaron que este fenómeno se debía primordialmente a la capacidad de E6*I de unirse a E6 de longitud completa, así como a la ubiquitin ligasa E6AP, ocasionando una menor degradación de la proteína p53 (Pim y Banks, 1999).

Por otro lado E6 de VPH-16 se une y acelera la degradación de procaspasa 8, la unión de la forma E6* a esta procaspasa, resulta en su estabilización. Por lo cual, se argumenta que el balance entre la estabilización y desestabilización de procaspasa 8, mediada por E6 y las isoformas E6*, respectivamente, contribuye a la alteración de la respuesta celular a los miembros de la superfamilia TNF. Así, la relación entre los niveles de expresión de E6 y E6*, podría ser un factor en la modificación de vías apoptóticas extrínsecas del hospedero (Filippova *et al*., 2007; Tungteakkhun *et al*., 2010).

Pocos han sido los estudios que se enfocan en investigar la contribución de las proteínas E6* durante el proceso carcinogénico, en uno de ellos se demostró la capacidad de E6*I de VPH-18 sobre la estabilidad de Akt, Dlg, MAGI-1, MAGI-2 y Scribble. Los resultados demostraron que la expresión de E6*I promueve una disminución en la expresión de Akt, Dlg, y Scribble en ausencia de la proteína E6 de longitud completa. Para el caso de Dlg, su disminución dada por E6* es independiente de la transcripción, y no se requiere de la interacción directa entre estas dos proteínas. A partir de estos hallazgos, se concluyó que las oncoproteínas E6 de los papilomavirus de alto riesgo, pueden marcar ciertos sustratos ya sea directa o indirectamente a través de proteínas E6*, cooperando en su degradación (Pim *et al*., 2009).

Numerosos estudios han mostrado que la presencia de las proteínas E6 y E7 de los papilomavirus de alto riesgo, es suficiente para inducir la inmortalización de queratinocitos humanos primarios, mientras que las proteínas de los VPHs de bajo riesgo no inducen inmortalización (McLaughlin-Durbin y Münger, 2009).

El primer blanco de E6 en ser identificado, y el más estudiado a la fecha, es p53. El supresor de tumores p53 es un factor de transcripción que coordina vías de señalización claves en la célula que le permiten enfrentar y reparar daños inducidos por estrés geno-citotóxico (Mantovani y Banks, 2001). Normalmente, p53 está presente en bajos niveles y es transcripcionalmente inactivo, el daño celular dispara un incremento en los niveles de la proteína p53 y su activación vía modificaciones post-traduccionales.

Una vez activada, p53 promueve el encendido de vías de señalamiento relacionadas a la reparación del ADN, arresto del ciclo celular y/o apoptosis. Además del daño genotóxico y citotóxico, p53 también puede ser activado por la estimulación imprevista de la síntesis de DNA, como la inducida por el VPH (Thomas *et al*., 1999).

El principal mecanismo por el cual los VPH de alto riesgo inactivan a p53, es por la inducción de su degradación a través la vía la ubiquitina. E6 de los VPH-AR inducen la degradación de p53 por medio de la formación de un complejo con la proteína celular asociada a E6 (E6AP), una ubiquitin ligasa E3, también conocida como UBE3A. E6AP pertenece a la familia con el dominio HECT de ubiquitin ligasas, cuyos dominios N-terminal divergentes son los que median el reconocimiento a sustratos. E6AP *per sé*, es incapaz de unirse a p53 e inducir su degradación, puesto que es necesario que E6 se una en primera instancia al dominio de reconocimiento a sustrato N-terminal de E6AP, antes de que p53 sea unido y ubiquitinado (Beaudenon y Huibregtse, 2008). Las proteínas E6 de los tipos de AR son capaces de unirse a la región central o *core* de p53, mediante la cual son capaces de inducir la degradación de p53 (Thomas *et al*., 1999).

El PBM sobre E6 confiere a esta proteína la capacidad de interactuar con proteínas que contienen dominios PDZ, estas proteínas se encuentran en sitios de unión célula-célula, y dan la polaridad al tejido. Estas proteínas pueden actuar como andamios en la transducción de señales. Miembros de esta familia de proteínas, como lo son MUPP, hDLG, y hSCRIB son marcadas para su degradación por la proteína E6 influyendo en el proceso carcinogénico inducido por papiloma virus (Banks et al., 2012).

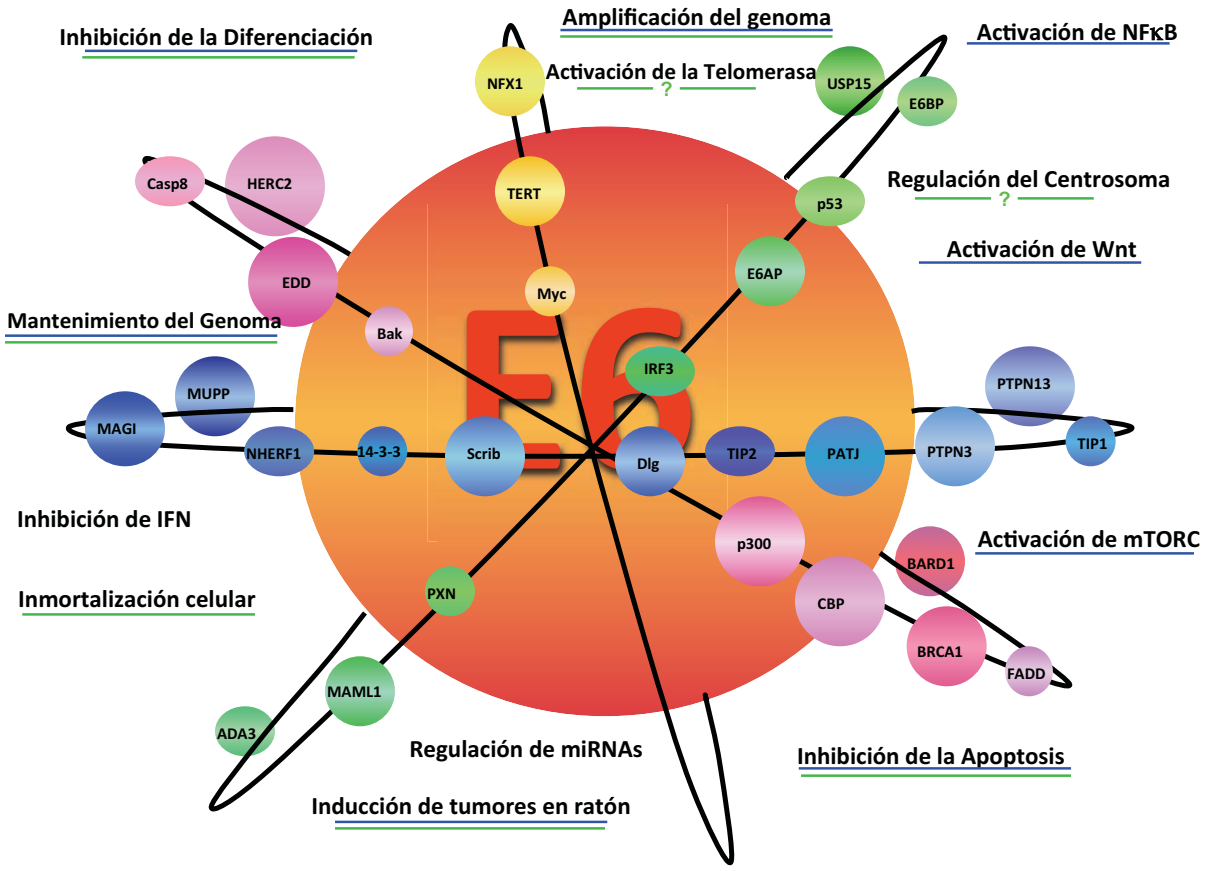


***Figura 3*. Blancos celulares de interacción para E6 y sus actividades biológicas asociadas.** E6 interactúa con múltiples blancos celulares a través de motivos bien definidos como LxxLL (por ejemplo: E6AP, MAML1 y PXN [en verde]) o PDZ (como por ejemplo: Dlg, Scrib y MAGI [azul]). Otros blancos celulares interactúan con E6 mediante motivos que no han sido completamente definidos. No obstante E6 perturba una gran variedad de funciones celulares, aquellas que están asociadas a las interacciones con los dominios LxxLL o PDZ están subrayadas en verde o azul, respectivamente.

Las proteínas de los papiloma virus poseen capacidades que regulan una amplia gama de funciones, que en conjunto, promueven la desregulación de mecanismos de control celular. La figura 3 resume algunos de los blancos celulares y los procesos que son regulados por E6.

ANTECEDENTES

La expresión de la proteína E6 es importante en el establecimiento de la carcinogénesis viral, dicha proteína es capaz de interactuar con diversos blancos celulares y modular su función. Recientemente Rozeblantt-Rosen *et al.* (2012) demostraron los resultados de un interactoma empleando proteínas de virus oncogénicos mediante ensayos de doble híbrido. Dentro de los blancos celulares potenciales de interacción con E6 se encontró a Sorting Nexin 27 (SNX27). En este listado de proteínas identificadas buscamos nuevos blancos celulares específicos para la proteína E6* de VPH 18 y encontramos a Vps26A (*Vacuolar protein sorting*), Vps35, Zixina y FHL-2, que de acuerdo a los resultados deberían interactuar únicamente con la forma E6* de VPH18.

La proteína FHL-2 (*Four and a Half LIM domains protein 2*) es una proteína de 32.2 KDa, la cual está constituida por cuatro y medio dominios LIM. La expresión de FHL-2 en tejidos ocurre mayoritariamente en corazón y ovario y en menor proporción en cerebro, pulmón, hígado, riñón e intestino. Las funciones ejercidas por esta proteína son variadas y redundantes pues ha sido implicada en procesos de diferenciación, supervivencia celular, adhesión, motilidad, transcripción y transducción de señales (Johannessen *et al*., 2006). La proteína FHL-2 se descubrió inicialmente como un gen sub-expresado en células de rabdomiosarcoma. Sin embargo, una expresión elevada de FHL-2 se ha detectado en otro tipo de neoplasias que incluyen, carcinoma hepatocelular, glioblastoma, próstata, ovario y cáncer gastrointestinal (Lin y Cheung, 2009). A la fecha no existen reportes acerca del estatus de expresión de FHL-2 en cáncer de cérvix por lo que resulta importante investigar el rol de esta proteína en la carcinogénesis inducida por VPH.

Por otro lado, reportes anteriores han demostrado que E6 y E6* del VPH-16 son capaces de interactuar y modular la actividad de la procaspasa 8 (Filippova *et al*., 2007; Tungteakkhun *et al*., 2010) por lo que resulta interesante evaluar la capacidad de

diferentes tipos de VPH de modular las actividades de procaspasa 8. Procaspasa 8 pertenece a una familia de cisteín-proteasas que promueven el corte a nivel de un residuo de aspartato de lo que deriva su nombre (cisteinil-aspartato proteasas). Las caspasas están implicadas en diversos procesos celulares entre los que destaca la iniciación de la apoptosis. Procaspasa 8, pertenece al grupo de caspasas iniciadoras las cuales se caracterizan por poseer un dominio efector de muerte (DED) que le permite interactuar con otras moléculas que regulan su activación (McIlwain, 2013).

Dentro de los potenciales blancos de interacción para E6 reportados por Rozeblantt-Rosen *et al.* (2012) se detectaron proteínas implicadas en el proceso de endocitosis y reciclaje celular: SNX27, Vps26A y Vps35. SNX27 pertenece a la familia de proteínas *Sorting*, de las cuales se conocen 33 miembros (Cullen, 2008), es importante recalcar el hecho de que SNX27 es el único miembro de la familia *Sorting* que posee un dominio PDZ lo cual lo hace un blanco ideal para su interacción con E6. Esta proteína esta implicada en tráfico y reciclaje de proteínas entre la membrana y el aparato de Golgi (Worby y Dixon, 2003) algunas de las cuales involucran el dominio PDZ (Valdés *et al.,* 2011).

La función más relacionada con VPH por algún miembro de la familia *Sorting* ha sido demostrada por el hecho de que SNX17 facilita el escape de la proteína de cápside viral L2 del endosoma tardío (Bergant *et al*., 2012) y este mecanismo está conservado entre los diferentes tipos de VPH (Bergant y Banks, 2013) lo cual sugiere que el virus utiliza la interacción con este tipo de proteínas durante el proceso infeccioso. Así mismo, la pérdida de esta proteína contribuye a un mal funcionamiento en la modulación del receptor de glutamato en el síndrome de Down (Wang *et al.,* 2013), siendo ésta el único reporte acerca de las implicaciones fisiopatológicas de la pérdida de SNX27.

Por otro lado, las proteínas Vps26 y Vps35 son miembros importantes del Retrómero, que es una estructura multiprotéica encargada de la selección de proteínas en el endosoma, para su reciclaje o bien para su degradación. El Retrómero comprende un

dímero de proteínas de la familia *Sorting* compuesto por una combinación, aún no definida, de SNX1, SNX2, SNX5 y SNX6 y un trímero de reconocimiento de proteínas llamado “cargo”, compuesto por Vps26, Vps29 and Vps35 (Bonifacio y Hurley, 2008). A pesar de que el dímero SNX es requerido para el reclutamiento del Retrómero a la membrana endosomal, la función de unión a “cargo” del Retrómero es realizada por el trímero mediante la unión de Vps35 a distintas proteínas “cargo” (Seaman, 2004).

La desregulación de la selección proteíca mediada por el Retrómero conlleva a varias patologías, incluyendo enfermedades neurodegenerativas como Alzheimer y paraplejia espástica; sin embargo, los mecanismos implicados en estos procesos aún no han sido determinados (Seaman, 2012). Es por ello que resulta relevante el estudio de las funciones de SNX27, Vps26 y Vps35 relacionadas con el ciclo viral y/o el proceso carcinogénico.

JUSTIFICACIÓN

El CaCu representa un serio problema de salud pública, principalmente en países en vías de desarrollo como el nuestro. A la fecha la infección persistente por el VPH esta siendo combatida con el advenimiento de las vacunas, que están encaminadas a disminuir la tasa de infección por este virus con la última finalidad de disminuir el número de casos de cáncer asociado a VPH. Sin embargo estas vacunas se han hecho accesibles a un número limitado de la población y los resultados de las campañas actuales de vacunación, así como el impacto en salud pública de estas vacunas, podrá evaluarse hasta dentro de varios años. Por otro lado, las vacunas existentes solo son profilácticas y brindan una protección limitada a un grupo pequeño de papilomavirus de alto y bajo riesgo, lo que deja abierta la posibilidad de que otros tipos virales aumenten su frecuencia de infección.

A pesar del gran conocimiento acerca del proceso infeccioso inducido por VPH, a la fecha, no se ha determinado el proceso carcinogénico completo inducido por el mismo, es por ello que resulta importante precisar aquellas actividades celulares que son moduladas por el VPH durante la infección o bien durante el proceso carcinogénico para que la comprensión de dichos eventos brinde la posibilidad de diseñar estrategias de detección, tratamiento y prevención eficaces.

Es de nuestro interés evaluar nuevos blancos celulares regulados por la proteína E6 en términos de interacción y relevancia biológica con el fin de entender la historia natural de la infección por el VPH y su progresión hacia cáncer.

HIPOTESIS

- Las proteínas E6 y E6* de los VPH de alto riesgo interactúan con diversos elementos celulares que regulan apoptosis, vías de reciclaje y procesos transcripcionales.

OBJETIVO GENERAL

- Determinar la capacidad de E6 y E6* de distintos tipos de VPH de interactuar con nuevos elementos celulares y determinar la relevancia biológica de dichas interacciones.

OBJETIVOS ESPECIFICOS

- Determinar la capacidad de interacción entre E6 y los nuevos blancos celulares: procaspasa 8, FHL-2 y SNX27 *in Vitro* e *in Vivo*.

- Determinar el efecto de la expresión de E6*I sobre proliferación y apoptosis en líneas celulares.

- Analizar el efecto de E6 sobre la distribución y localización de caspasa 8, FHL-2 y SNX27 en células positivas y negativas a VPH.

- Determinar el efecto de E6 sobre los elementos de la vía de reciclaje endosomal.

MATERIALES Y MÉTODOS

Cultivos celulares

Las líneas celulares HeLa, Hek-293,HaCaT y U2OS fueron mantenidas en medio Eagle modificado de Dulbecco (DMEM) suplementado con penicilina/estreptomicina (concentración) suero bovino fetal al 10%,a 37°C en atmósfera húmeda al 5% de CO_2.

Plásmidos

Los plásmidos E6-HA utilizados fueron descritos previamente en Thomas et al., 2013. Los plásmidos de Zixina, FHL-2, Vps25 y SNX27 fueron donados por los doctores: Thomas Hofmann (DKFZ), vonZastrow (DKFZ), Heidi McBride (McHill University) y Martin Payford (NHI USA) e Isabel Mérida (Instituto de Biotecnología de Barcelona) respectivamente. Los plásmidos E6*ΔC-VPH18 y pcDNA3-Caspasa8 fueron creados mediante técnicas de PCR convencionales. Los plásmidos codificantes para las diferentes proteínas E6 unidas al tag HA están descritas en Thomas *et al*., 2013.

Expresión y purificación de proteínas de fusión GST-E6

La expresión y purificación de las diferentes GST-E6 fue realizada de acuerdo a Pim et al., 2009.

Traducción in Vitro

La proteínas procaspasa 8, DED, Zixina, FHL-2, SNX27 y las diferentes E6 fueron traducidas y marcadas con [^{35}S]-Cisteína ó [^{35}S]-Metionina *in Vitro* empleando el sistema T7-TNT in Vitro Translation system (Promega) de acuerdo a las instrucciones del fabricante utilizando 1ug de plásmido, se emplearon inhibidores de RNAsas (Promega). La traducción de las proteínas fue comprobada mediante PAGE y autorradiografía.

Ensayos de interacción in Vitro

Cantidades iguales de las proteínas GST-E6 fueron incubadas con procaspasa 8, DED, Zixina, FHL-2 ó SNX27 traducidas *in Vitro* durante 90 minutos en buffer PBS 1X/0.2%

Tritón a 4°C ó temperatura ambiente, según el caso. Así mismo se utilizó un 20% de la proteína traducida *in Vitro* como input. Transcurrido el tiempo de incubación se realizaron 3 lavados con PBS 1X/0.2% Tritón. Una vez eliminada la mayoría del buffer de lavado se agregaron 20 μL de buffer de carga a cada muestra y se llevo a cabo PAGE. Los geles fueron secados y revelados mediante autorradiografía.

Ensayos de degradación in Vitro

Cantidades iguales de las proteínas procaspasa 8, DED, FHL-2 y SNX27 traducidas *in Vitro*, fueron incubadas en presencia o ausencia de proteínas E6 traducidas *in Vitro* durante 30, 60 y 90 minutos a 30°C. Transcurrido el tiempo de incubación, toda la muestra fue cargada en un gel y sometida a PAGE, el gel fue secado y revelado mediante autorradiografía. Se empleó el 20% de cada condición al tiempo cero como control de carga.

Western Blot

600,000 células Hek-293 fueron sembradas en placas p100, se permitió la adherencia y una vez alcanzada una confluencia del 70% las células fueron transfectadas con los plásmidos con procaspasa 8, SNX27 y FHL-2 solos o en combinación con los diferentes E6. 24 hrs. postransfección las células fueron cosechadas y se realizó un extracto total de proteína añadiendo simplemente buffer de carga. Los niveles de proteína fueron sometidos a PAGE y analizados con anticuerpos específicos: anti-HA (1:500), anti-caspasa 8 (1:1000), anti SNX27 (1:10000), anti-FHL2 (1:1000) y anti b-galactosidasa (1:5000), los cuales fueron detectados mediante anticuerpos secundarios anti-ratón o anti-conejo, según fuera el caso, acoplados a la peroxidasa de rábano (HRP). El plásmido LacZ fue empleado como control de transfección en una concentración de 200 ng.

Inmunoprecipitación

600,000 células Hek-293 fueron sembradas en placas p100, una vez alcanzado el 70% de confluencia, las células fueron transfectadas con plásmidos E6-HA y 24 hrs. postransfección fueron cosechadas y la proteína fue extraída en dos fases, soluble e insoluble utilizando buffer RIPA (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Sodium

Deoxycholate y 1 mM EDTA). El lisado fue sometido a centrifugación (13,000 rpm/10 min) y la fase soluble fue incubada con perlas anti-HA (Easy view affinity gel) durante 2 hrs. Transcurrido el tiempo de incubación las muestras fueron lavadas 3 veces con buffer RIPA y finalmente se agregaron 20 μL de buffer de carga. Las muestras fueron sometidas a PAGE y los niveles de proteínas fueron analizados mediante western blot con anticuerpos anti-HA, anti-SNX27 (Abcam) y anti FHL-2 (Sigma) según el caso.

Ensayos de apoptosis por Anexina V

Células Hek-293 fueron transfectadas con procaspasa 8 sola o en combinación con los diferentes E6. 24 hrs. postransfección las células fueron cosechadas y procesadas con el kit Anexin V Staining Kit Fluorescein (Roche) de acuerdo a las instrucciones del fabricante. Las muestras fueron analizadas mediante FACS y los resultados fueron presentados en una gráfica de barras representando el promedio de tres experimentos independientes indicando la desviación estándar. La realizó una prueba de *t-student* para determinar la significancia entre las diferentes condiciones.

Inmunofluorescencia

Células U2OS fueron transfectadas con los plásmidos pcDNA3-caspasa8, p-myc-FHL-2, pGFP-SNX27, HA-E6 de los diferentes tipos virales utilizados por medio de lipofección. 24 hrs. postransfección las células fueron fijadas en solución de PBS 1X/4% formaldehído durante 30 minutos, después de varios lavados las células fueron incubadas con anticuerpos dirigidos para detectar las proteínas Caspasa 8, SNX27, FHL-2 y tag-HA durante 2 hrs. Siguiendo una serie de lavados las células fueron incubadas con anticuerpos secundarios anti-ratón y anti-conejo acoplados a los fluoróforos Alexa 488 (verde) ó Alexa 594 (rojo). Las células fueron montadas en portaobjetos con medio de montaje Becton 1X y analizadas en el microscopio de fluorescencia Leica DMLBcon una cámara Leica (A01M871016) ó con un microscopio confocal Zeiss LSM 510 con dos laseres que generan líneas de excitación a 480 y 510 nm. Las imágenes fueron recolectadas bajo el objetivo de inmersión 60X.

Silenciamiento de E6 y fraccionamiento celular en células HeLa

Células HeLa fueron sembradas en cajas p60, cuando alcanzaron confluencia del 60%, las células fueron transfectadas con siRNA (Darmacon) dirigidos contra E6/E7 (5´ CAUUUACCAG CCCGACGAG) ó Luciferasa que fue empleado como control utilizando RNAiMAX Lipofectamine (Invitrogen) de acuerdo a las instrucciones del fabricante. 72 hrs. post-silenciamiento las células fueron cosechadas para extraer proteína total o realizar fraccionamiento celular con el Sub-cellular Proteome Extraction Kit (Calbiochem) de acuerdo a las instrucciones del fabricante y los niveles de proteína evaluados mediante western blot; o bien fueron procesadas para inmunofluorescencia empleando anticuerpos anti-FHL-2, -SNX27, -HA, Vps35, -Caspasa 8, -Golgi, -Myc, y –GFP según fuera el caso. Se empleó anti-p53 (DO1) para detectar aquellas células en las que el silenciamiento fue exitoso. Todos los anticuerpos fueron utilizados en una concentración 1:50.

RESULTADOS

Búsqueda de nuevos blancos celulares para la proteína E6 de los virus del papiloma Humano

Una vez identificados los blancos potenciales de interacción con la proteína E6 del VPH procedimos a comprobar si estas proteínas se unían a E6 y a E6*.

PROCASPASA 8

E6 y E6* de diferentes tipos de VPH interactúan con procaspasa 8

Por medio de ensayos de interacción *in Vitro*, se demostró la capacidad de la proteína E6 de diferentes VPH para interactuar con la procaspasa 8. Se incluyeron los tipos virales 16, 18 y 11. Las diferentes proteínas E6 de tamaño completo y el producto E6* acopladas a GST fueron incubadas con procaspasa 8 traducida *in Vitro* y marcada radiactivamente con [^{35}S]-Metionina a temperatura ambiente y a 4ºC, para reflejar las condiciones previamente reportadas (Filippova *et al*., 2007). La figura 4 muestra que todas las proteínas E6 analizadas tienen la capacidad de interactuar con procaspasa 8; sin embargo, se observa una interacción mayor para la proteína E6* de VPH-18. Así mismo observamos que la unión a procaspasa 8 es sensible a la temperatura, dado que la señal disminuye considerablemente a 4ºC (Panel derecho).

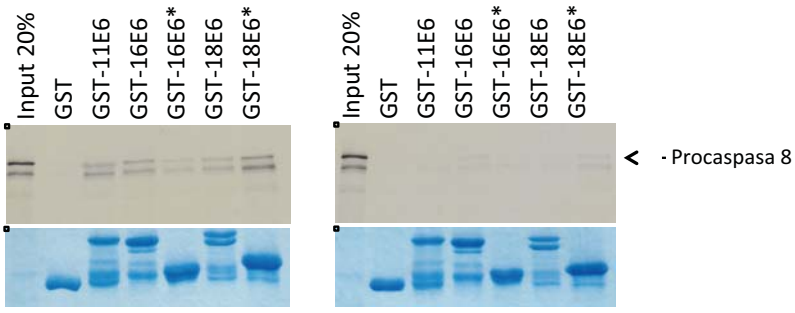


Figura 4. Las proteínas E6 de diferentes tipos de VPH interactúan con Procaspasa 8. Procaspasa 8 fue traducida y marcada con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT in Vitro translation Kit (Promega) de acuerdo a las instrucciones del fabricante. La proteína traducida *in Vitro* fue incubada con proteínas de fusión GST-E6 de los diferentes VPH durante 90 minutos a temperatura ambiente

y a 4ºC. Después de lavados, las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía (panel superior). El panel inferior corresponde a la tinción de Coomassie de las proteínas GST-E6 empleadas. Se utilizó el 20% de la proteína traducida *in Vitro* como input. El panel izquierdo representa las condiciones de interacción a temperatura ambiente mientras que el panel derecho corresponde a el experimento realizado a 4ºC.

La unión entre procaspasa 8 y E6 es dependiente del DED

El DED regula la activación de procaspasa 8 mediante su interacción con diferentes proteínas. Para determinar si la unión de E6 con procaspasa 8 ocurre a través del dominio efector de muerte, se repitieron los ensayos de interacción *in Vitro* utilizando el DED traducido *in Vitro*. Nuevamente los resultados demostraron una capacidad similar de interacción entre las proteínas E6 y el DED, el cual fue nuevamente, sensible a la temperatura (Fig. 5)

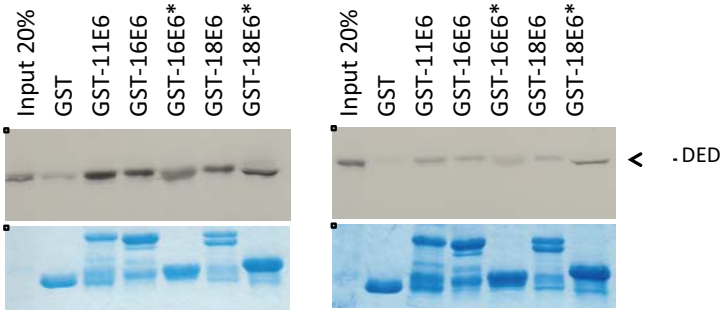


Figura 5. Las proteínas E6 de diferentes tipos de VPH interactúan con Procaspasa 8 a través del dominio efector de muerte (DED). El DED fue traducido y marcado con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT *in Vitro* translation Kit (Promega) de acuerdo a las instrucciones del fabricante. La proteína traducida *in Vitro* fue incubada con proteínas de fusión GST-E6 de los diferentes VPH durante 90 minutos a temperatura ambiente y a 4ºC. Después de lavados, las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía (panel superior). El panel inferior corresponde a la tinción de Coomassie de las proteínas GST-E6 empleadas. Se utilizó el 20% de la proteína traducida *in Vitro* como input. El panel izquierdo representa las condiciones de interacción a temperatura ambiente mientras que el panel derecho corresponde a el experimento realizado a 4ºC.

La interacción de E6* de VPH-18 con procaspasa 8 es dependiente del dominio C-terminal

Estudios previos han demostrado una capacidad diferencial entre E6 y E6* de VPH-16 para unirse con procaspasa 8 (Filoppova *et al*., 2007). En nuestros análisis notamos una capacidad incrementada de E6* de VPH-18 para interactuar con procaspasa 8 comparada con la proteína completa. Para determinar el mecanismo por medio del cual podría estar sucediendo, repetimos los ensayos de interacción empleando una forma

silvestre de E6* de VPH-18 y una mutante con el Dominio C-terminal eliminado (E6*ΔC). Como se puede observar en la figura 6 E6* interactúa mejor con procaspasa 8 comparada con la forma completa E6, así mismo, se observa que esta capacidad requiere de los últimos 8 aminoácidos de E6* en la región C-terminal.

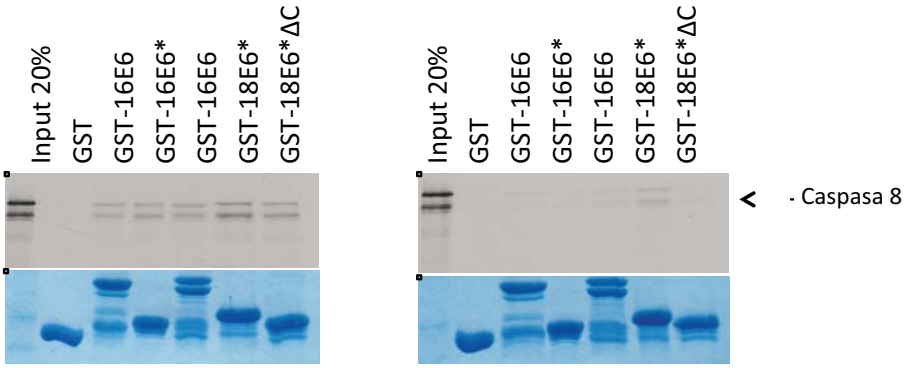


Figura 6. E6* de VPH-18 requiere de la porción C-terminal para su interacción con procaspasa 8. Procaspasa 8 fue traducida y marcada con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT *in Vitro* translation Kit (Promega) de acuerdo a las instrucciones del fabricante. Procaspasa 8 fue incubada con proteínas de fusión GST-E6 de los diferentes VPH durante 90 minutos, a temperatura ambiente y a 4ºC. Se incluyó el GST-E6* Δ C al cuál se le eliminaron los últimos 12 aa correspondientes a la región C-terminal. Después de lavados, las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía (panel superior). El panel inferior corresponde a la tinción de Coomassie de las proteínas GST-E6 empleadas. Se utilizó el 20% de la proteína traducida *in Vitro* como input. El panel izquierdo representa las condiciones de interacción a temperatura ambiente mientras que el panel derecho corresponde a el experimento realizado a 4ºC.

E6 induce activación de caspasa 8

Normalmente los blancos celulares que interactúan con E6 son marcados para su degradación vía proteosoma por lo que nos interesamos en evaluar si E6 inducía degradación de procaspasa 8. En una serie de ensayos de degradación in Vitro, encontramos que ninguna de las proteínas E6 utilizadas (VPH-11, 16 y 18), así como las formas estrella de VPH-16 y 18, fueron capaces de inducir algún cambio en la estabilidad de procaspasa 8 a los 60 minutos (Fig. 7). Debido a que no se observó ningún cambio en los niveles de procaspasa 8 decidimos repetir el análisis y extenderlo a 3 horas incluyendo los E6 de los VPH-11 y 31, p53 fue utilizado como control en ambos casos. Los resultados mostraron que a las 3 horas, ninguna de las proteínas E6 utilizadas fue capaz de reducir los niveles de procaspasa 8 (datos no mostrados).

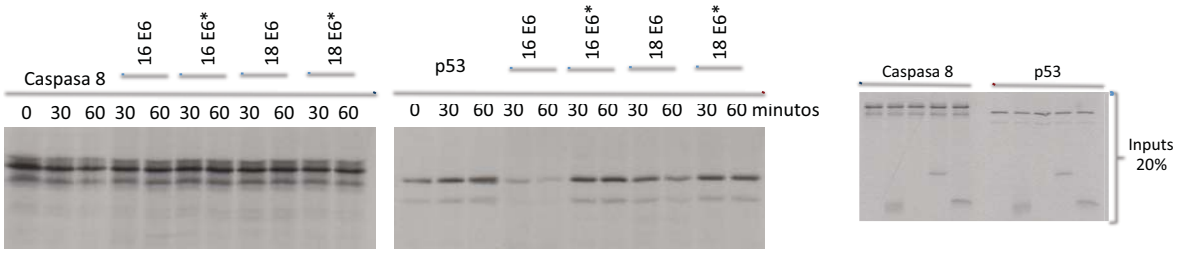


Figura 7. E6 no induce degradación de Procaspasa 8 *in Vitro*. Procaspasa 8, p53 y E6 de los diferentes VPH fue traducidos y marcados con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT in Vitro translation Kit (Promega) de acuerdo a las instrucciones del fabricante. Cantidades iguales de procaspasa 8 y p53 fueron incubadas solo o en combinación con los diferentes E6 durante una hora a 30ºC para inducir degradación de procaspasa 8. Transcurrido el tiempo de incubación las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía. Se utilizó el 20% de la reacción como input, al tiempo 0.

Los resultados demostraron que E6 no tiene ningún efecto sobre los niveles de procaspasa 8 indicando que no existe degradación de la misma en el sistema *in Vitro*. Por lo que procedimos a evaluar los efectos de E6 sobre los niveles de procaspasa 8 *in Vivo*. Células Hek-293 fueron transfectadas con plásmidos que expresan procaspasa 8 solo o en combinación con E6 que poseen el tag HA de los VPH tipo 11, 16, 18 y 18*. 24 hrs. postransfección las células fueron cosechadas y los niveles de caspasa 8 evaluados mediante western blot. Como se puede observar en la figura 8 los niveles de caspasa 8 activa (p18) se incrementan cuando las células son transfectadas con E6 de los VPH 11, 16 y 18, así como para la forma E6*I de VPH-18.

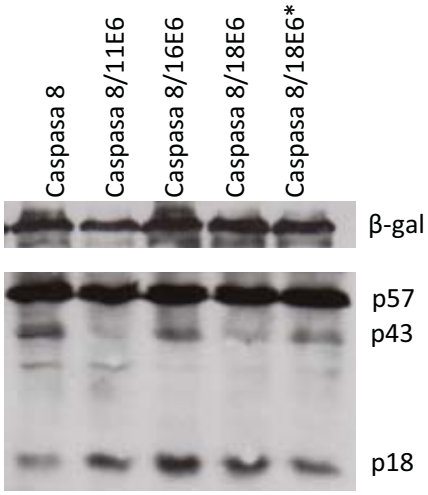


Figura 8. E6 induce activación de caspasa 8 en células Hek-293. Células 293 fueron transfectadas con Procaspasa 8 sola o en combinación con plásmidos que expresan E6 de los VPH tipo 11, 16 y 18, así como E6* de VPH-18. 24 hrs. postransfección las células fueron cosechadas y se obtuvo proteína total adicionando únicamente buffer de carga. Los niveles de proteína fueron evaluados mediante western blot. Lac-Z fue empleado como control de transfección.

La unión de E6 a procaspasa 8 no induce apoptosis

Caspasa 8 es activada por señales de muerte iniciando así la vía intrínseca de apoptosis. Por lo tanto nos dimos a la tarea de realizar ensayos de apoptosis para evaluar si la activacion de caspasa 8 mediada por E6 que se observó *in Vivo* es suficiente para aumentar los niveles de apoptosis en células que sobreexpresan caspasa 8. Células Hek-293 fueron transfectadas con procaspasa 8 en presencia o ausencia de E6 y E6* de VPH-18, 24 hrs. después los niveles de apoptosis fueron evaluados mediante la tecnica de anexina V. Los resultados muestran que la expresión de E6 no tiene efectos significativos en los niveles de apoptosis inducidos por caspasa 8 (Fig. 9), indicando que la activación de caspasa 8 inducida por E6 (Fig. 8) no resulta en un incremento de la apoptosis.

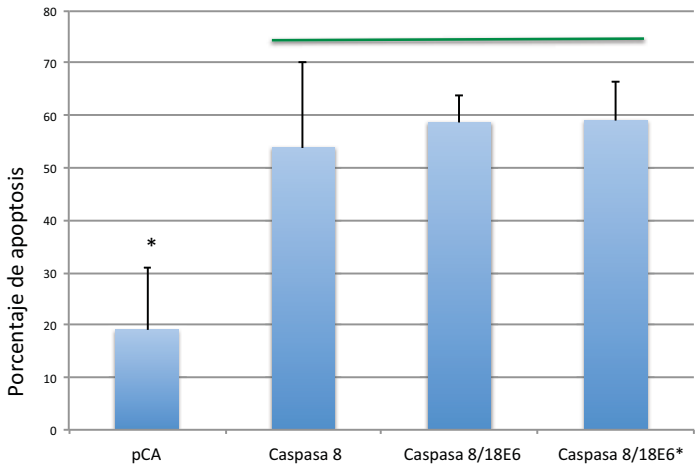


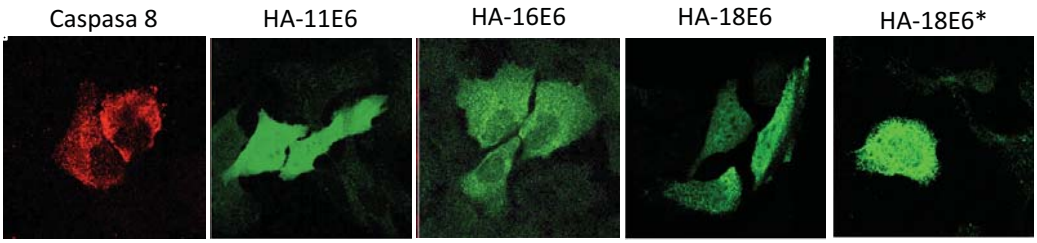
Fig. 9. La activación de caspasa 8 mediada por E6 no es suficiente para inducir apoptosis. Células 293 fueron transfectadas con procaspasa 8 sola o en combinación con E6 o E6* de VPH18. 24 hrs. postransfección las células fueron cosechadas y procesadas con el kit Annexin V Binding Kit Fluorescein de acuerdo a las instrucciones del fabricante. Las muestras fueron analizadas mediante FACS. La gráfica representa el promedio de tres experimentos independientes con la respectiva desviación estandar.(*p≤0.05). La linea verde indica que no hay diferencia significtiva entre esas condiciones.

E6 induce translocación de caspasa 8 a núcleo

Estudios previos han demostrado que la activación de caspasas es un paso importante en el ciclo de vida del virus (Moody *et al*., 2007; Terenzi *et al*., 2008; Yu *et al*., 2007). Las caspasas se localizan primordialmente en el citoplasma (Zhivotovsky *et al*., 1999) mientras que E6 se expresa tanto en citoplasma como en núcleo. Por lo cual evaluamos la capacidad de E6 de modular la localización celular de caspasa 8. Para hacer esto, células U2OS fueron transfectadas con procaspasa 8 sola o en combinación con los diferentes E6 marcados con HA. 24 hrs. postransfección las células fueron fijadas con formaldehido al 4% y procesadas para inmunofluorescencia y

analizadas para detectar la expresión de E6-HA y caspasa 8 empleando anticuerpos anti-HA (Roche) y anti caspasa 8 (Cell signaling) seguidos de anticuerpos secundarios acoplados a Alexa 488 y Rodamina, respectivamente. Caspasa 8 muestra una distribución citoplasmática cuando es transfectada sola (Fig. 10A) mientras que E6 se expresa en toda la célula (Fig. 10A). En contraste cuando se analizó la expresión de caspasa 8 en células co-transfectadas con cualquiera de las proteínas E6 empleadas se observa un grado de co-localización entre E6 y caspasa 8 (Fig. 10B). Adicionalmente, se observa que en las células en que E6 tiene una localización nuclear, hay un aumento de caspasa 8 en núcleo, correspondiente a la señal de E6 (Fig. 10B). Los resultados fueron conservados entre los E6 de los VPH 11, 16 y 18 así como con E6* de VPH-18 demostrando que E6 puede inducir activación de caspasa 8 con un consecuente aumento en la localización nuclear de la misma.

10A)



10B)

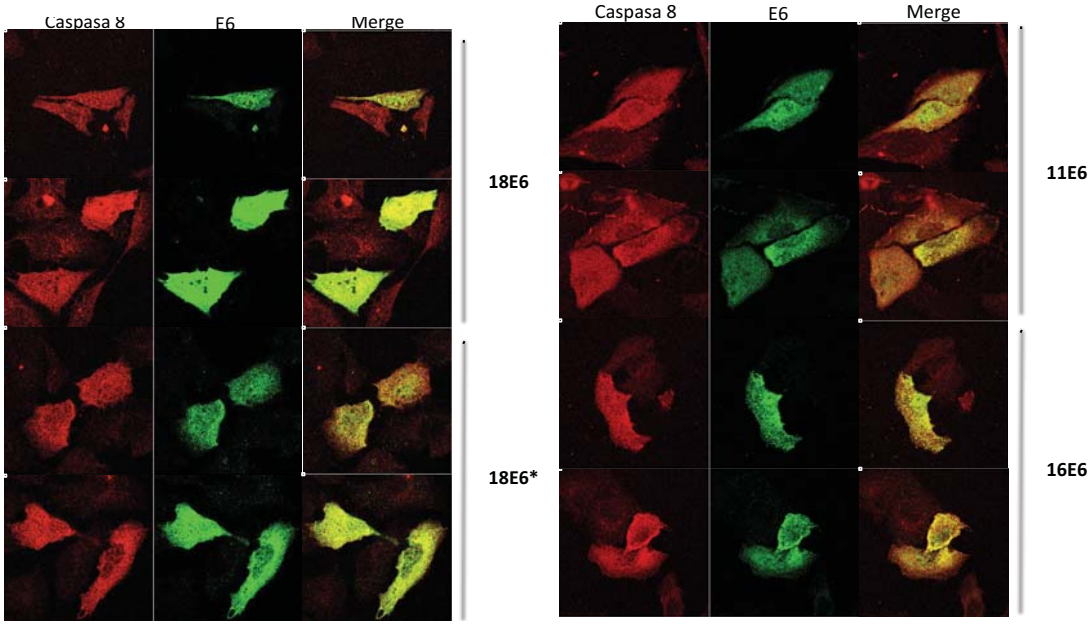


Figura 10. E6 induce translocación de caspasa 8 al núcleo. Células U2OS fueron transfectadas con Procaspasa 8 sola o en combinación con plásmidos que expresan HA-E6 de los VPH tipo 11, 16 y 18, así como la forma E6* de VPH-18. 24 hrs. postransfección las células se fijaron con PBS/4% formaldehído durante 20 minutos posteriormente fueron incubadas con los anticuerpos anti-caspasa 8 (1C12) (Cell signaling) y anti HA (Roche) durante 1 hora seguidas de anticuerpos secundarios anti Rabbit y anti Mouse acoplados a Alea 594 y Alexa 488, respectivamente. Las laminas fueron observadas en el microscopio confocal con laser de excitación a 480 y 510 nm. Las imágenes fueron capturadas con el objetivo de inmersión 60X.

La presencia de caspasa 8 en el núcleo de células HeLa es dependiente de E6

Para determinar de que manera el patrón de caspasa 8 se veía afectado por E6 de VPH-18 endógeno en células HeLa, las células HeLa fueron silenciadas con el uso de siRNA específicos para E6 y un siRNA dirigido contra Luciferasa fue usado como control. Después de 72 hrs. post-silenciamiento las células fueron cosechadas y se obtuvieron, mediante fraccionamiento, las fracciones citoplasmática, membranal, citoesquelética y nuclear, los niveles de proteína fueron evaluados mediante western blot. Los resultados demostraron una fuerte acumulación de p53 en la fracción nuclear de células silenciadas para E6/E7. En el caso de caspasa 8 activa, esta fue encontrada en el núcleo de células HeLa (Fig. 11A) la cual se ve disminuida cuando E6 es silenciada. Así mismo, se llevó a cabo un análisis de inmunofluorescencia para caspasa 8 en las células HeLa silenciadas para E6 y Luciferasa; p53 fue utilizado como control. En el mismo experimento se incluyeron células HaCaT como control de comparación. Los resultados demuestran que la señal de caspasa 8 en células HeLa es nuclear y una vez silenciado E6 hay una acumulación de caspasa 8 en el citoplasma (Fig. 11B).

11A)

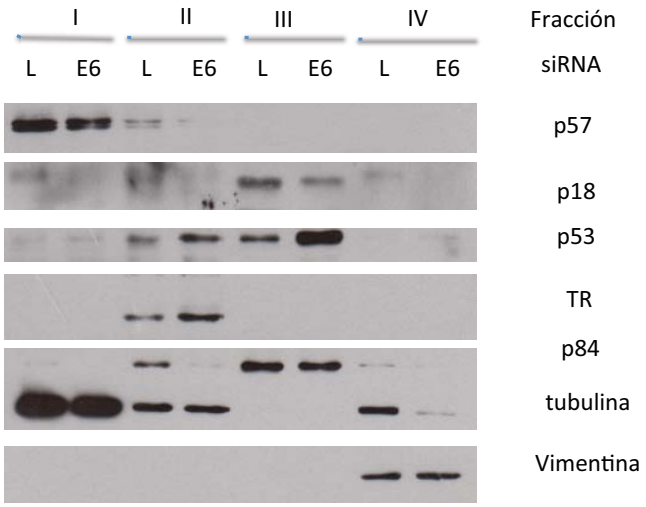
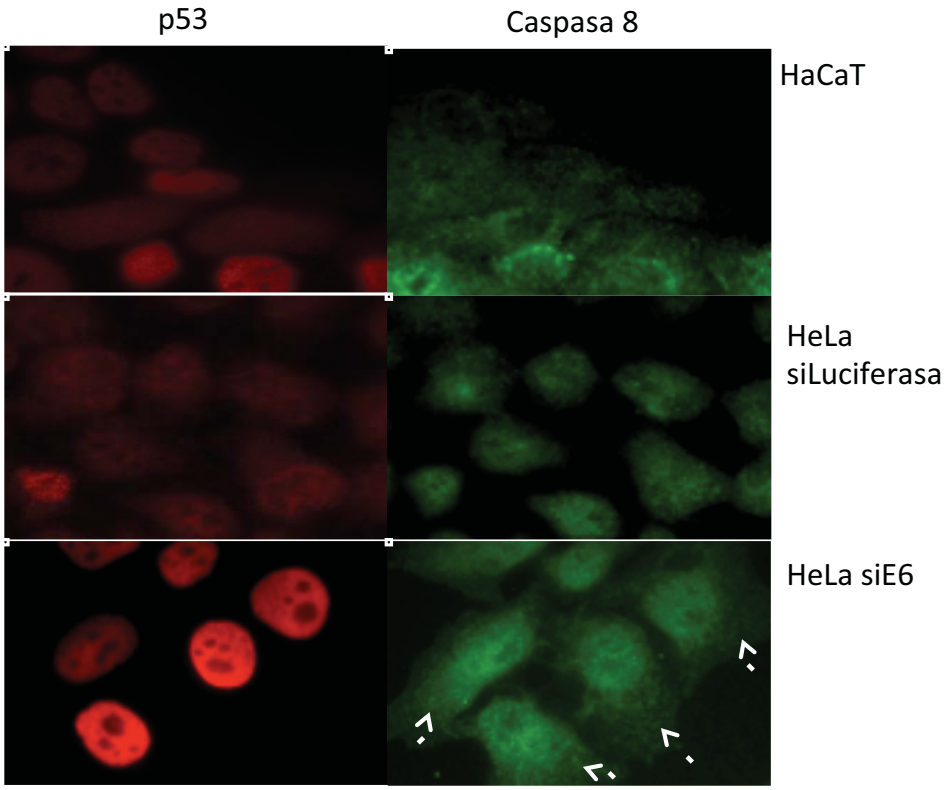


Figura 11. E6 de VPH-18 induce expresión de caspasa 8 en el núcleo de células HeLa. Panel A) Las células fueron transfectadas con siRNA dirigidos contra Luciferasa (L) o E6/E7 (E6) y después de 72 horas las células fueron cosechadas y la proteína fue extraída en las fracciones: citoplasma, membrana, núcleo y citoesqueleto. Las fracciones fueron sometidas a western blot para analizar caspasa 8 (completa p57 y activa p18) y p53. Los

marcadores de carga y de fraccionamiento fueron vimentina, tubulina, p84 y receptor de transferrina. Panel B) Las células fueron transfectadas con siRNA dirigidos contra Luciferasa (L) o E6/E7 (E6) y después de 72 horas las células fueron fijadas y teñidas para caspasa 8 y p53. Las células HaCaT fueron incluidas como comparación. El ensayo de inmunofluorescencia muestra una fuerte acumulación nuclear de p53 posterior al silenciamiento de E6/E7 y la correspondiente incremento de caspasa 8 en el citoplasma (indicado con las flechas).

11B)



Por otra parte quisimos extender la capacidad de caspasa 8 de interactuar con E6 de otros tipos virales. Para hacer esto, se llevaron a cabo ensayos de interacción *in Vitro* empleando caspasa 8 traducida *in Vitro* y E6 de los VPH: 31, 33, 51 y 58, acopladas a GST. Los resultados demostraron que la capacidad de E6 de interactuar con procaspasa 8 está conservada para los diferentes tipos virales (datos no mostrados).

E6 de VPH de alto riesgo interactúan con FHL-2

Dentro de los blancos potenciales de interacción para VPH-18 E6* encontramos a FHL-2, Zixina, Vps26A y Vps35. Para determinar el efecto biológico de dichas interacciones procedimos a comprobar la capacidad de unión entre VPH-18 E6* y las proteínas antes mencionadas mediante ensayos de interacción *in Vitro*, empleando a E6 de los VPH-16 y 18 como controles.

Sorpresivamente, y contrario a lo reportado por Rozenblatt *et al.,* (2012) la forma estrella de VPH-18 no fue capaz de interactuar con Zixina o Vps26A, mostrando una capacidad de interacción muy débil con estas proteínas (datos no mostrados). Por otra parte La proteína FHL-2 demostró una alta capacidad de interacción con las formas completas de E6 (VPH-16 y 18) no así cuando se trataba de la forma E6*, nuevamente contrastando con el reporte de Rozenblatt-Rosen *et al*., (Fig. 12)

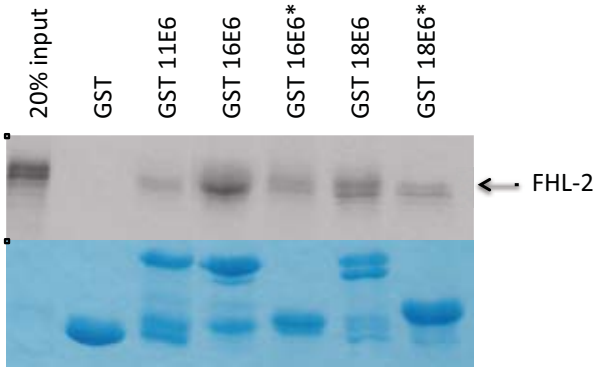


Figura 12. E6 de los VPH de alto riesgo interactúan con la proteína FHL-2. FHL-2 fue traducida y marcada con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT in Vitro translation Kit (Promega) de acuerdo a las instrucciones del fabricante. La proteína fue incubada con proteínas de fusión GST-E6 de los diferentes VPH durante 90 minutos a temperatura ambiente. Después de varios lavados, las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía (panel superior). El panel inferior corresponde a la tinción de Coomassie de las proteínas GST-E6 empleadas. Se utilizó el 20% de la proteína traducida *in Vitro* como input. Nótese que la interacción de FHL-2 con E6 de VPH-11 es muy débil comparada con los E6 de alto riesgo.

La interacción de FHL-2 es específica para E6 y no interactúa con E7

FHL-2 es una proteína que funciona como transmisor molecular vinculando varias vías de señalización mediante la regulación transcripcional y su pérdida ha sido vinculada a

un progreso más severo en distintos tipos de cáncer. Estudios previos han demostrado que E7 de VPH-16 modula la actividad transcripcional de FHL-2 (Campo-Fernández *et al.,* 2007) por lo cual quisimos comprobar si la capacidad de FHL-2 de interactuar con E6 se conservaba con E7. Para demostrarlo repetimos los ensayos de interacción in Vitro empleando las proteínas E6 y E7 unidas a GST. Los resultados demostraron una clara interacción entre FHL-2 y E6, sin embargo, cuando FHL-2 es incubada con E7 no se observa interacción entre las proteínas (Fig. 13) lo cual contrasta con los datos reportados previamente. Al mismo tiempo se realizó una inmunofluorescencia en células U2OS transfectadas con plásmidos que expresan FHL-2 y HA-E6 de VPH-18. Los resultados muestran que cuando las células son co-transfectadas con ambos plásmidos existe co-localización entre dichas proteínas (Datos no mostrados).

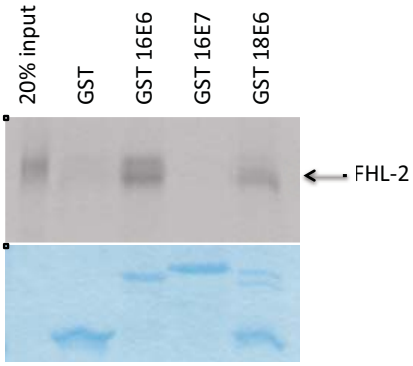


Figura 13. FHL-2 interactúa específicamente con E6 y no con E7 de los VPH de alto riesgo. FHL-2 fue traducida y marcada con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT in Vitro translation Kit (Promega) de acuerdo a las instrucciones del fabricante. La proteína fue incubada con proteínas de fusión GST-E6 de VPH 16 y 18 y GST-E7 de VPH-16 durante 90 minutos a temperatura ambiente. Después de varios lavados, las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía (panel superior). El panel inferior corresponde a la tinción de Coomassie de las proteínas GST-E6 empleadas. Se utilizó el 20% de la proteína traducida in Vitro como input. La interacción es específica para E6.

E6 de VPH-18 induce disminución de los niveles de FHL-2 en células HeLa

E6 induce degradación de sus blancos celulares a través del proteosoma, debido a que FHL-2 interactúa con E6 quisimos averiguar si esta interacción era capaz de promover degradación de FHL-2 en células HeLa donde E6 es expresada continuamente. Para determinar esto, células HeLa fueron silenciadas con siRNA específicos para E6 y siLuciferasa fue empleado como control. 72 hrs. post-silenciamiento los niveles de proteína evaluados mediante western blot. Los resultados demostraron que los niveles de FHL-2 están disminuidos en las células HeLa los cuales se recuperan cuando E6 es silenciado (Fig. 14). Sin embargo, en las células en las que E6AP fue silenciado no se observan cambios en los niveles de FHL-2, lo cual nos indica que E6 esta utilizando

otro método de inducción de degradación de FHL-2 en células HeLa. Por otra parte se realizaron ensayos de inmunofluorescencia para evaluar los niveles de FHL-2 antes y después del silenciamiento de E6 y corroborarlos con los resultados de western blot. La figura 15 muestra una clara recuperación de los niveles de FHL-2 en el citoplasma de células que han sido silenciadas para E6, p53 fue analizado como control del silenciamiento.

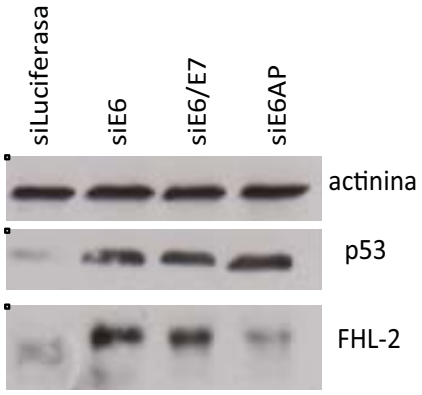


Figura 14. E6 reduce los niveles de FHL-2 en células HeLa. Las células fueron transfectadas con siRNA dirigidos contra Luciferasa, E6/E7 o E6AP y después de 72 horas las células fueron cosechadas y la proteína fue extraída adicionando buffer de carga. Los niveles de proteína fueron evaluados mediante western blot para analizar FHL-2 y p53. Obsérvese la recuperación en los niveles de p53 después del silenciamiento de E6/E7 y E6AP. Los niveles de FHL-2 se recuperaron únicamente en las células en que se silenció E6 mostrando que la regulación en los niveles de FHL-2 en células HeLa depende únicamente de E6.

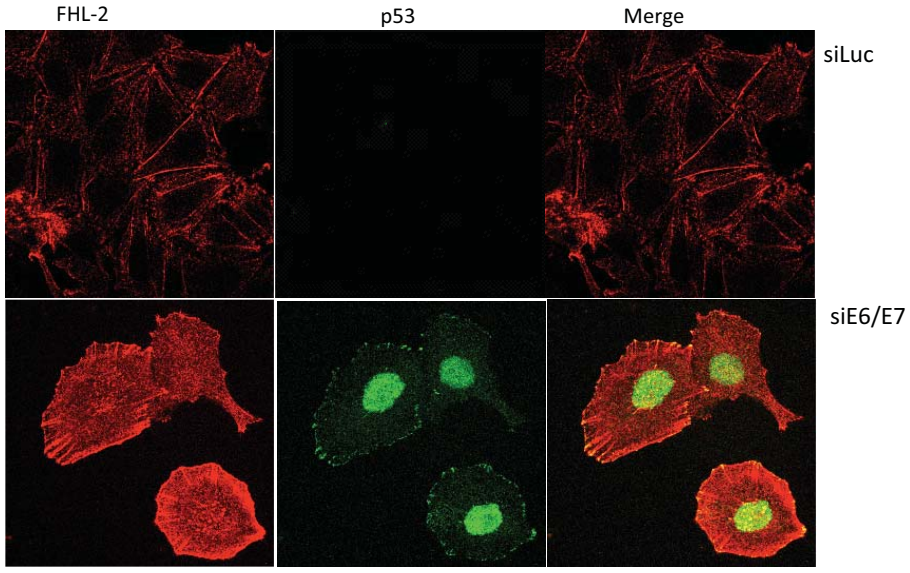


Figura 15. E6 modula la distribución celular de FHL-2 en células HeLa. Las células fueron transfectadas con siRNA dirigidos contra Luciferasa, E6/E7 y después de 72 horas las células fueron fijadas y teñidas para FHL-2 y p53. Obsérvese la recuperación nuclear de p53 después del silenciamiento de E6/E7. Los niveles de FHL-2 se recuperaron en el citoplasma cuando E6 fue silenciado

Para determinar la participación del proteosoma en la regulación de los niveles de FHL-2 mediados por E6, células HeLa fueron tratadas con el inhibidor Z-Leu-Leu-Leu (CBZ) (Sigma) y procesadas para inmunofluorescencia. Los resultados demostraron un incremento en la señal de FHL-2 citoplásmica en aquellas células que fueron tratadas con el inhibidor comparadas con aquellas células que solo fueron tratadas con el vehículo (Fig.16), así mismo se observa un incremento en p53, el cual fue empleado como control. Estos resultados son similares a los obtenidos cuando se silencia E6 con la aparición de estructuras filamentosas características de FHL-2.

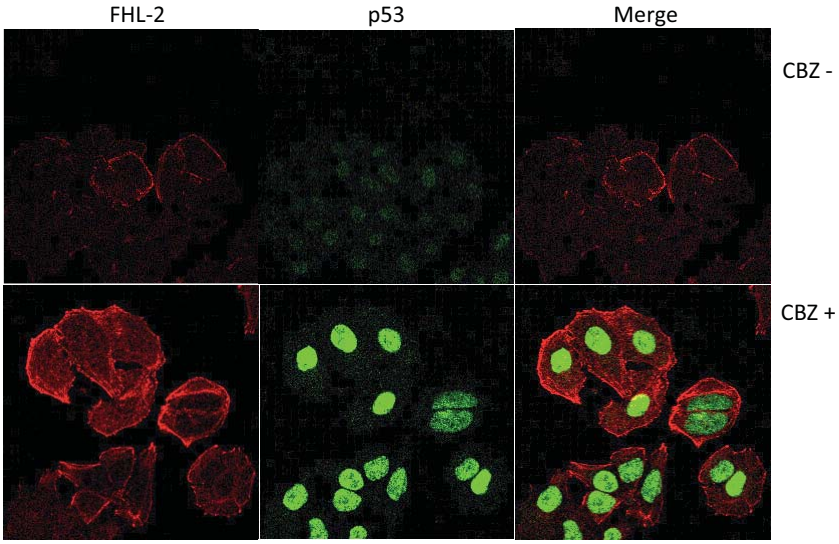


Figura 16. Los niveles de FHL-2 en células HeLa son dependientes del proteosoma. Células HeLa fueron tratadas con inhibidores de proteosoma (CBZ) durante 3 horas, posteriormente las células fueron fijadas y teñidas para FHL-2 y p53. Obsérvese la recuperación nuclear de p53 después del tratamiento. Los niveles de FHL-2 se recuperaron en el citoplasma.

Siguiendo la misma dirección, procedimos a realizar un ensayo de fraccionamiento celular en células HeLa silenciadas para E6 y verificar en que fracción se recupera la proteína y si esta, corresponde a lo observado en los ensayos de inmunofluorescencia. Los resultados mostraron una consistencia entre lo observado en las imágenes de inmunofluorescencia tanto con el silenciamiento (Fig. 15) como cuando se usó el inhibidor de proteosoma (Fig. 16). Los niveles de proteína se recuperaron en las fracciones citoplasmática y membranal cuando la expresión de E6 fue inhibida, así mismo se observa una recuperación en los niveles de p53 en las células silenciadas para E6, el cual fue empleado como control (Fig. 17).

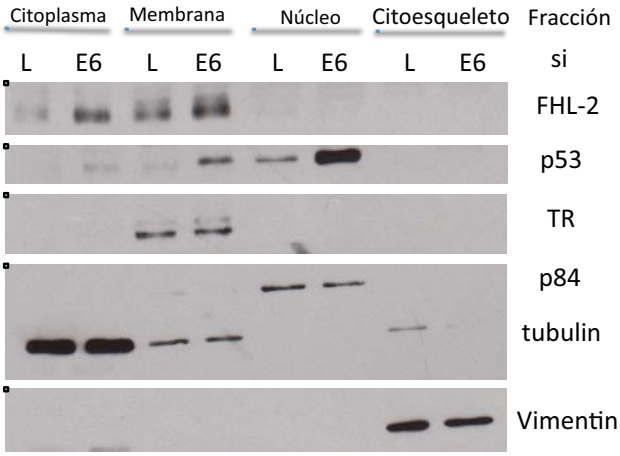


Figura 17. E6 de VPH-18 modula la distribución celular de FHL-2 en células HeLa. Las células fueron transfectadas con siRNA dirigidos contra Luciferasa (L) o E6/E7 (E6) y después de 72 horas las células fueron cosechadas y la proteína fue extraída en las fracciones: citoplasma, membrana, núcleo y citoesqueleto. Las fracciones fueron sometidas a western blot para analizar FHL-2 y p53. Los marcadores de carga y de fraccionamiento fueron vimentina, tubulina, p84 y receptor de transferrina. Se muestra una fuerte acumulación nuclear de p53 posterior al silenciamiento de E6/E7 y la correspondiente incremento de FHL-2 en membrana y citoplasma.

Los resultados anteriores posicionan a la proteína FHL-2 como un nuevo blanco celular de interacción para las proteínas E6 de los VPH de alto riesgo y al mismo tiempo demuestran que E6 induce degradación de esta proteína mediante el proteosoma pero sin involucrar a E6AP, probablemente E6 se une a otra ubiquitin ligasa y facilita la degradación de FHL-2.

SORTING NEXIN 27 (SNX27)

SNX27 interactúa con E6 de VPH 16 y 18 mediante el dominio PDZ

Una de las proteína celulares que fueron detectadas en el interactoma realizado por Rozenblatt-Rosen *et al*., (2012) fue Sorting Nexin 27, dicha proteína fue de particular atención ya que dentro de la familia Sorting Nexin es la única que posee un dominio PDZ los cuales son reconocidos por E6 mediante su motivo de unión a dominios PDZ, el PBM. SNX27 es una proteína implicada en el proceso de maduración de Endosoma a Lisosoma, regulación de las vías de tráfico celular así como polaridad celular en Linfocitos. Para comprobar si E6 de los VPH de alto riesgo son capaces de reconocer e interactuar con SNX27 se realizaron ensayos de interacción *in Vivo* empleando proteína SNX27 traducida *in Vitro* y E6 de los VPH-16 y 18 acopladas a GST. Los

resultados mostraron una clara interacción entre las proteínas E6 de los VPH de alto riesgo y SNX27 (Fig. 18).

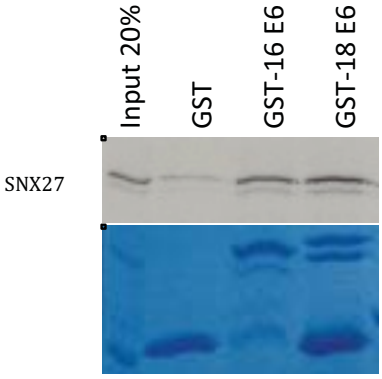


Figura 18. E6 interactúa con Sorting Nexin 27. Sorting Nexin 27 fue traducida y marcada con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT in Vitro translation Kit (Promega) de acuerdo a las instrucciones del fabricante. SNX27 fue incubada con proteínas de fusión GST-E6 de los VPH 16 y 18 durante 90 minutos a temperatura ambiente. Después de varios lavados, las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía (panel superior). El panel inferior corresponde a la tinción de Coomassie de las proteínas GST-E6 empleadas. Se utilizó el 20% de la proteína traducida *in Vitro* como input.

Para determinar si el PBM de E6 es el responsable de esta interacción repetimos los ensayos de interacción in Vitro en los cuales incluimos una proteína E6 con una mutación T156Q la cual, elimina la capacidad de E6 de interactuar con los dominios PDZ (Boon y Banks, 2013). La figura 19 muestra que la mutación T156Q exibe una reducción en la capacidad de E6 para reconocer a SNX27 indicando que dicha interacción es específica para E6 y está mediada por el PBM.

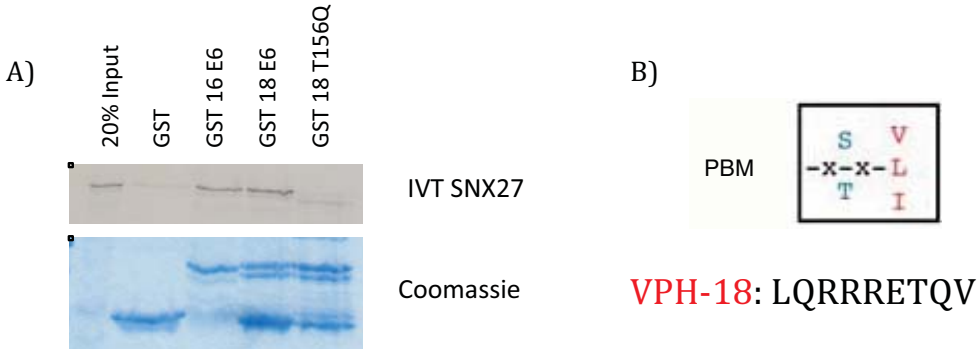


Figura 19. E6 interactúa con Sorting Nexin 27 mediante el dominio PDZ. Panel A) Sorting Nexin 27 fue traducida y marcada con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT in Vitro translation Kit (Promega) de acuerdo a las instrucciones del fabricante. SNX27 fue incubada con proteínas de fusión GST-E6 de los VPH 16 y 18 durante 90 minutos a temperatura ambiente. Así mismo se empleó una mutante de E6 en el PBM, GST-E6T156Q. Después de varios lavados, las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía (panel superior). El panel inferior corresponde a la tinción de Coomassie de las proteínas GST-E6 empleadas. Se utilizó el 20% de la proteína traducida *in Vitro* como input. Se observa que GST-E6T156Q no es capaz de interactuar don SNX27. Panel B) Representación gráfica del PBM en E6 de VPH-18, se muestra el sitio consenso PBM para todos los VPH.

SNX27 interactúa con E6 *in Vivo*

Para comprobar si la interacción entre E6 y SNX27 sucede *in Vivo*. Células HEK-293 fueron transfectadas con plásmidos que expresan E6 unidos al tag HA de los VPH-16 y 18. 24 hrs. postransfección las células fueron cosechadas, la proteína extraída e incubada con perlas Easy view Red anti-HA (Invitrogen) que reconocen y precipitan proteínas con el epítopo HA, después de varios lavados la proteína fue analizada mediante PAGE y western blot. Los resultados obtenidos comprueban que la interacción de SNX27 con E6 *in Vitro* se conserva en la célula como se puede observar en la figura 20 Cuando E6 es inmunoprecipitado se detecta a SNX27.

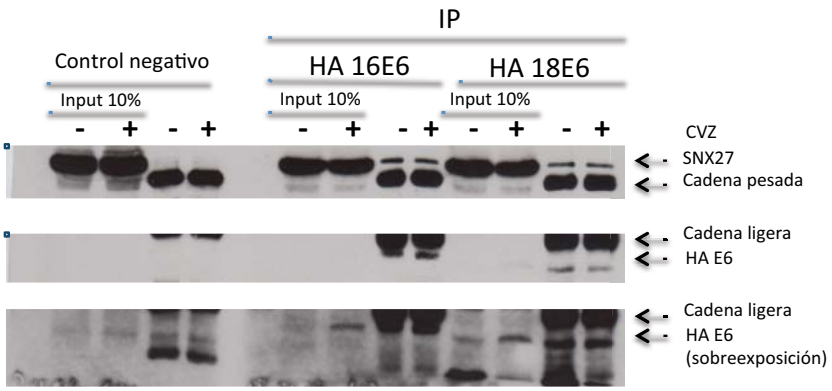


Figura 20. E6 interactúa con SNX27 *in Vivo*. Células Hek-293 fueron transfectadas con plásmidos que expresan E6 de los VPH tipo 16 y 18 unidos al tag HA. 24 hrs. postransfección las células fueron cosechadas y se obtuvo proteína utilizando el buffer E1A. La fracción soluble fue incubada con perlas HA Easy view Red (Sigma) durante dos horas, posteriormente las perlas fueron lavadas y sometidas a western blot para analizar SNX27 y HA-E6. Para incrementar la cantidad de proteína E6 y favorecer la interacción, se emplearon inhibidores de proteosoma durante tres horas antes de la cosecha de las células. Se observa que SNX27 co-inmunoprecipita con E6. El panel inferior corresponde a una sobreexposición del film con el objetivo de detectar la señal correspondiente a E6.

De igual manera se realizaron ensayos de inmunofluorescencia en células U2OS transfectadas con GFP-SNX27 y HA-E6 de VPH-18 así como E6*, la cual carece del PBM, como control. La figura muestra a SNX27 con un patrón de expresión en forma vesicular mientras que E6 se expresa en toda la célula (Fig. 21, panel superior). Por otro lado, cuando las células son co-transfectadas con SNX27 y HA-E6 se observa una co-localización de ambas proteínas en un patrón que es correspondiente con SNX27, en forma de vesículas (Fig. 21 panel inferior). En el caso de E6* no se observa co-

localización con SNX27 lo cual corresponde a que la interacción observada esta mediada por el dominio PDZ y el PBM en E6.

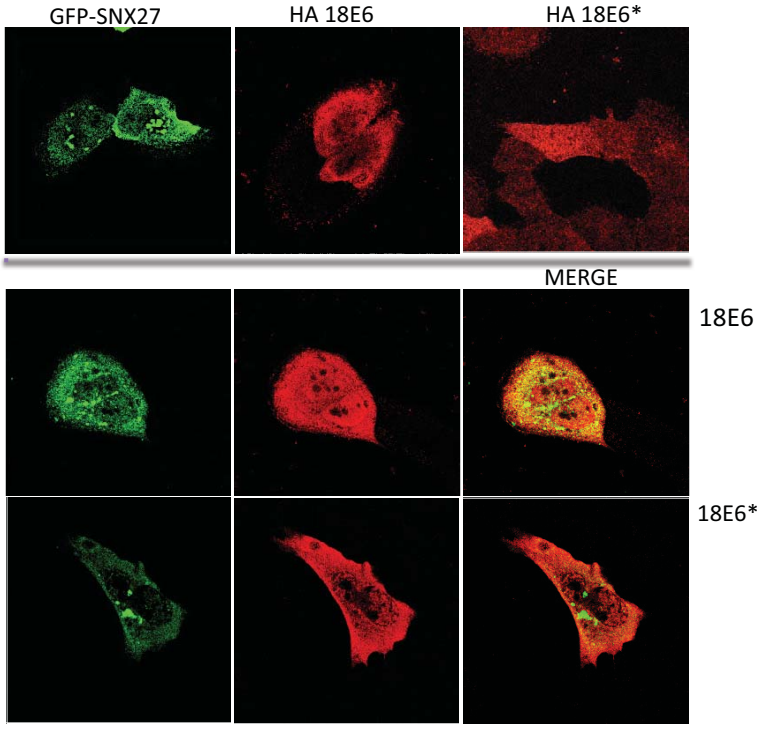


Figura 21. E6 co-localiza con SNX27. Células U2OS fueron transfectadas con SNX27 sola o en combinación con plásmidos que expresan HA-E6 y HA-E6* de VPH-18. 24 hrs. postransfección las células se fijaron con PBS/4% formaldehído durante 20 minutos posteriormente fueron incubadas con los anticuerpos anti-SNX27 (Abcam) y anti HA (Roche) durante 1 hora seguidas de anticuerpos secundarios anti Mouse y anti Rabbit acoplados a Alexa 488 y Alexa 594, respectivamente. Las láminas fueron observadas en el microscopio confocal con laser de excitación a 480 y 510 nm. Las imágenes fueron capturadas con el objetivo de inmersión 60X. Obsérvese el patrón vesicular dado por SNX27.

E6 no induce degradación de SNX27

Quisimos comprobar si la interacción de E6 con SNX27 inducía la degradación de esta última. Para comprobar esto, se llevaron a cabo ensayos de degradación in Vitro. Sorpresivamente encontramos que E6 de los VPH tipo 16 y 18 no inducen degradación de SNX27 a las 3 horas, al mismo tiempo incluimos una mutante de E6 con el PBM eliminado que mostró un comportamiento similar en cuanto a la degradación de SNX27 (Fig. 22). Se realizó un ensayo de degradación de p53 como control.

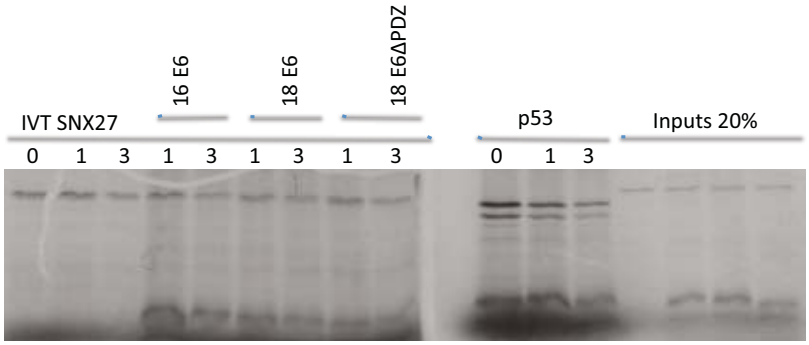


Figura 22. E6 no induce degradación de SNX27 *in Vitro*. SNX27, p53 y E6 de los diferentes VPH fue traducidos y marcados con [^{35}S]-Met *in Vitro*, utilizando el kit T7-TNT in Vitro translation Kit

(Promega) de acuerdo a las instrucciones del fabricante. Cantidades iguales de SNX27 y p53 fueron incubadas solas o en combinación con los diferentes E6 durante una hora a 30ºC para inducir degradación de SNX27. Transcurrido el tiempo de incubación las muestras fueron sometidas a PAGE. El gel fue secado y analizado mediante autorradiografía. Se utilizó el 20% de la reacción como input, al tiempo 0.

E6 modifica el patrón de localización de SNX27 pero no los niveles totales de la proteína en células HeLa

Quisimos evaluar de que manera un E6 endógeno altera los niveles de SNX27 en células HeLa. Para realizar esto, se silenció la expresión de E6 en células HeLa mediante RNA de interferencia específico para E6, E6AP o contra Luciferasa como control. 72 hrs. post-silenciamiento las células fueron cosechadas y los niveles de proteína determinados mediante western blot. Al mismo tiempo otro set experimental fue procesado para inmunofluorescencia empleando anticuerpos anti SNX27 (Abcam) y anti p53 (Santa Cruz) el cual funcionó como control de silenciamiento. La figura 23 muestra que los niveles totales de SNX27 no se ven alterados en presencia o ausencia de E6 o E6AP.

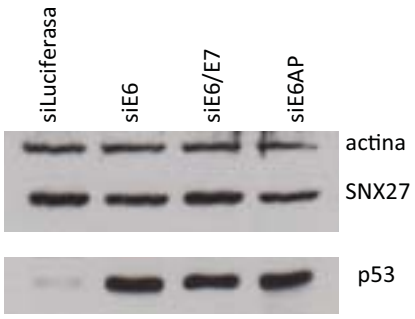


Figura 23. E6 no modifica los niveles de SNX27 en células HeLa. Las células fueron transfectadas con siRNA dirigidos contra Luciferasa, E6/E7 o E6AP y después de 72 horas las células fueron cosechadas y la proteína fue extraída adicionando buffer de carga. Los niveles de proteína fueron evaluados mediante western blot para analizar SNX27 y p53. Obsérvese la recuperación en los niveles de p53 después del silenciamiento de E6/E7 y E6AP. Los niveles de SNX27 no cambian cuando se silencia E6 ni E6AP.

Sin embargo, cuando se realizó el análisis de inmunofluorescencia notamos la aparición de estructuras vesiculares en aquellas células en las que la expresión de E6 fue inhibida (Fig. 24), dichas estructuras corresponden a la señal de SNX27 las cuales perecen acumularse alrededor del núcleo en una manera similar a Aparato de Golgi. Para verificar si las vesículas de SNX27 observadas en la figura 24 pertenecen o co-localizan en Golgi, células U2OS fueron transfectadas con GFP-SNX27 y teñidas para SNX27 y Golgi. Los resultados mostraron que SNx27 no co-localiza con el aparato de Golgi (Fig. 25), sin embargo estas vesículas parecen estar posadas cobre dicha

estructura celular lo cual indica que existe un proceso en células HeLa que esta siendo desregulado por la expresión de E6 involucrando a SNX27 y el aparato de Golgi como podría ser la vía de reciclaje y tráfico celular.

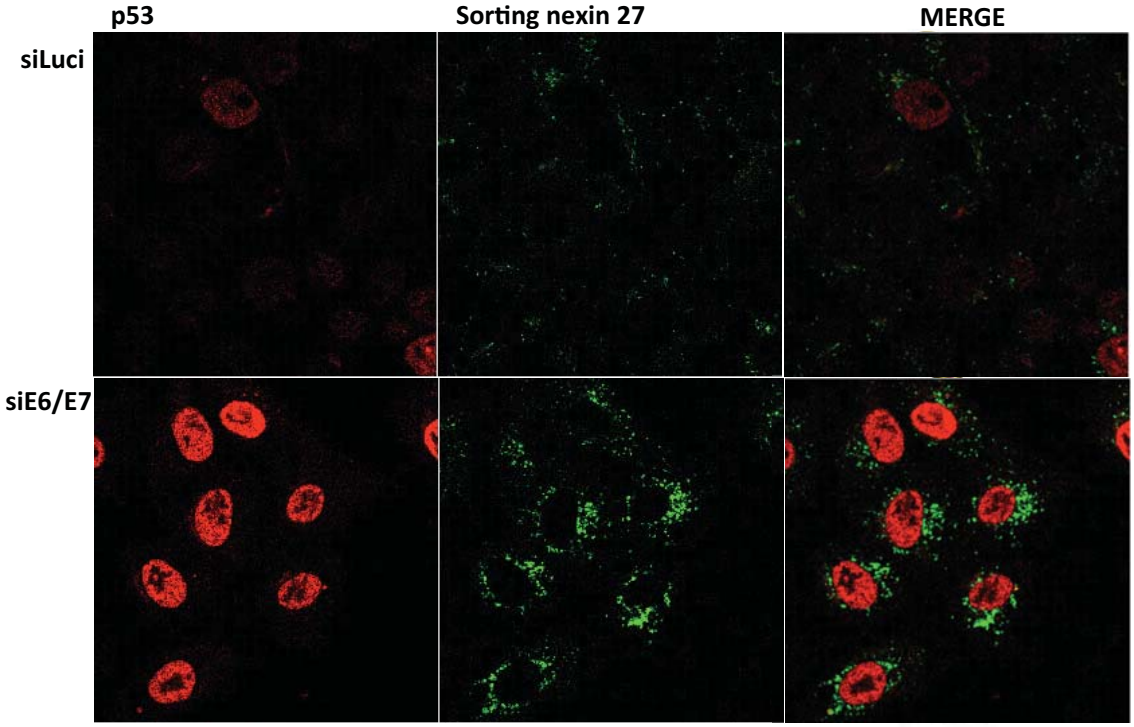


Figura 24. E6 modula la distribución celular de SNX27 en células HeLa. Las células fueron transfectadas con siRNA dirigidos contra Luciferasa, E6/E7 y después de 72 horas las células fueron fijadas y teñidas para SNX27 y p53. Obsérvese la recuperación de p53 en el núcleo de células silenciadas para E6. SNX27 se recupera en un patrón vesicular en las células silenciadas para E6.

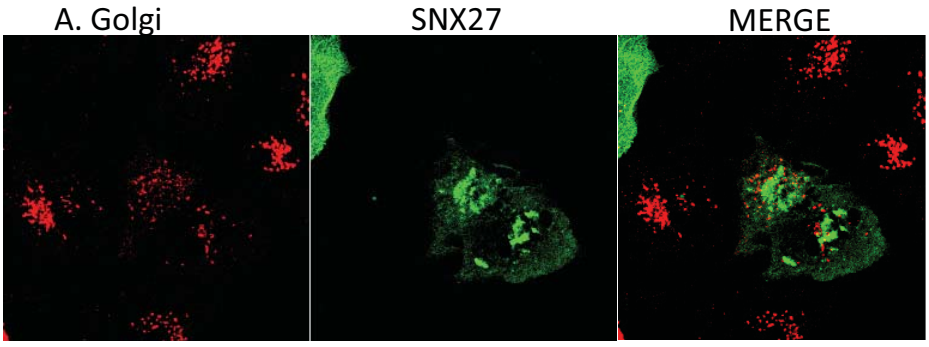


Figura 25. SNX27 no co-localiza en Aparato de Golgi. Células U2OS fueron transfectadas con GFP-SNX27. 24 hrs. postransfección las células se fijaron con PBS/4% formaldehído durante 20 minutos posteriormente fueron incubadas con los anticuerpos anti-GFP (Santa cruz)

y Golgin-97 (CDF4) (Molecular probes) durante 1 hora seguidas de el anticuerpo secundario anti Mouse acoplado a Alexa 594. Las laminas fueron observadas en el microscopio confocal con laser de excitación a 480 y 510 nm. Las imágenes fueron capturadas con el objetivo de inmersión 60X. Se observa que ambas señales comparten el espacio celular mas no co-localizan.

SNX27 modula los niveles de E6 en células HeLa

Debido a que el silenciamiento de E6 no parece tener efecto sobre los niveles de SNX27 nos preguntamos si de alguna manera SNX27 podría estar modulando la estabilidad de E6. Para llevar a cabo esto, se repitió el silenciamiento en células HeLa pero ahora utilizando un siRNA dirigido contra SNX27 y evaluando los niveles de E6 en un extracto total. Los resultados demostraron que en las células donde la expresión de SNX27 fue inhibida hay un decremento en los niveles de proteína E6 (Fig. 26) lo cual indica que de cierta manera, SNX27 estabiliza a E6 incrementa la estabilidad de E6.

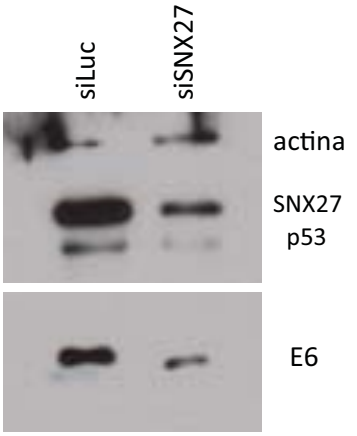


Figura 26. El silenciamiento de SNX27 afecta los niveles de E6. Las células fueron transfectadas con siRNA dirigidos contra Luciferasa (Luc) o SNX27 y después de 72 horas las células fueron cosechadas y la proteína fue extraída adicionando buffer de carga. Los niveles de proteína fueron evaluados mediante western blot para analizar SNX27 y p53. Obsérvese que los niveles de SNX27 disminuyen posterior al silenciamiento y los niveles de p53 no se ven afectados por SNX27. Los niveles de E6 se ven disminuidos en las células en las que se ha silenciado SNX27.

SNX27 es una proteína que regula la degradación de ciertas proteínas mediante el lisosoma, debido a que encontramos que de cierta manera SNX27 incrementa los niveles de E6 quisimos averiguar cual es el proceso celular mediante el que E6 reduce sus niveles. Para determinarlo, células HeLa fueron tratadas con inhibidores de proteosoma (CBZ) y con inhibidores de la vía endocítica (NH_4CL y Brefeldina A). Los resultados mostraron una recuperación en los niveles de E6 cuando las células son tratadas con los inhibidores de proteosoma y de transporte vesicular indicando que E6 es degradada tanto por vía proteosoma como por vía lisosomal (Fig. 27).

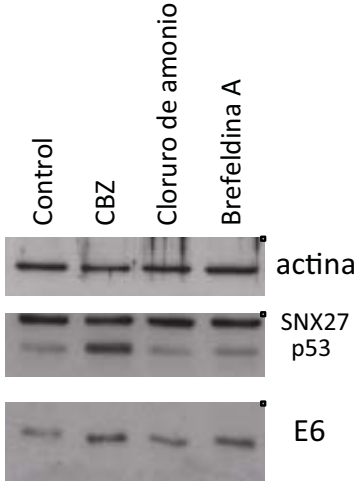


Figura 27. Los niveles de E6 son afectados por inhibidores de la vía endocítica en células HeLa. Las células HeLa fueron tratadas con inhibidores de la vía endocítica cloruro de amonio y brefeldina A, así como un inhibidor de proteosoma CBZ durante 3 horas, 15 minutos y 30 minutos respectivamente. Posteriormente las células fueron cosechadas y se obtuvo proteína total. Los niveles de proteína fueron evaluados mediante western blot para analizar SNX27, p53 y E6. Actina fue evaluada como control de carga. Obsérvese la recuperación en los niveles de E6 después del tratamiento con CBZ y BFA.

E6 evita la interacción de SNX27 con componentes del Retrómero

Uno de los datos que más llamo la atención es el hecho de que E6 evita que SNX27 se acumule en las estructuras vesiculares observadas en células HeLa. Debido a que SNX27 juega un papel importante en las vías de reciclaje celular procedimos a evaluar la presencia o ausencia de componentes del Retrómero, como Vps35, en estas estructuras en células HeLa. Los resultados muestran que los niveles de Vps35 no son dependientes de E6, sin embargo cuando E6 es silenciado y los niveles de SNX27 son incrementados, observamos una recuperación en la localización entre SNX27 y Vps35 (Fig. 28) indicando que E6 evita la interacción de SNX27 con componentes del Retrómero probablemente inhibiendo los procesos de reciclaje celular al mismo tiempo que evita su degradación vía lisosomal.

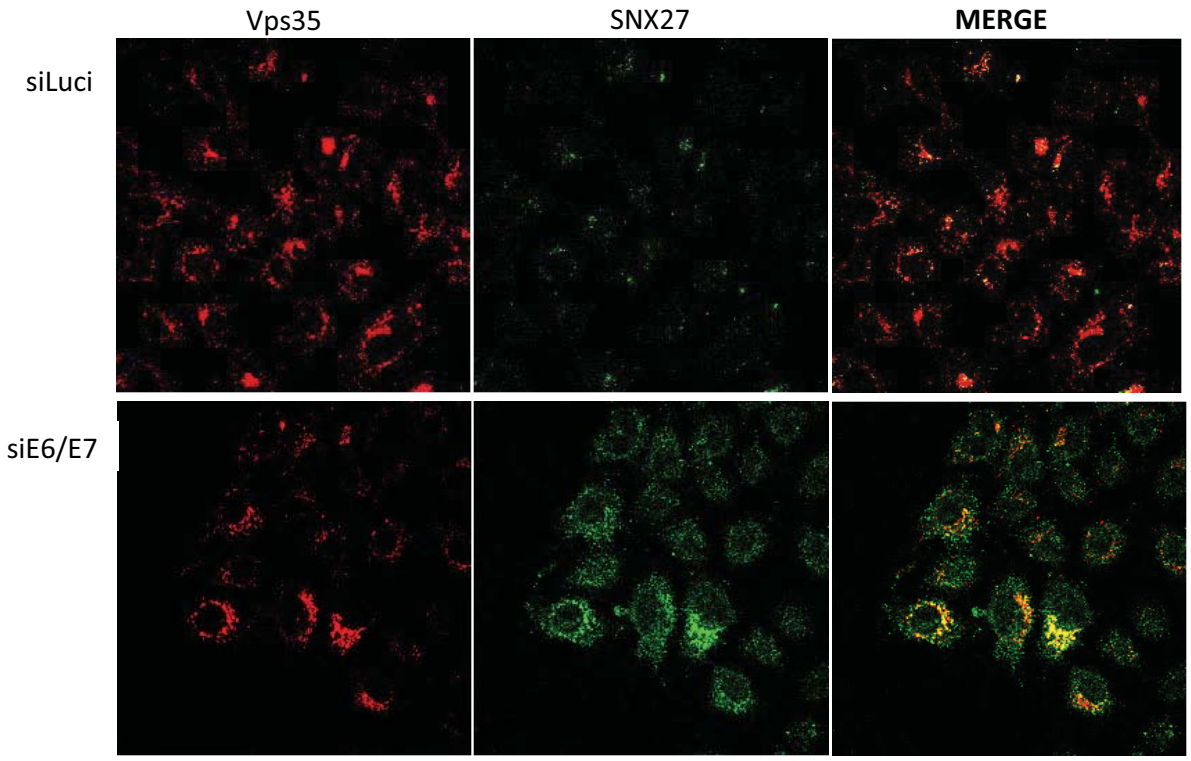


Figura 28. E6 modula la distribución celular de SNX27 en células HeLa. Las células fueron transfectadas con siRNA dirigidos contra Luciferasa y E6/E7 y después de 72 horas las células fueron fijadas y teñidas para SNX27 y Vps35. Como se puede observar en la figura los niveles de SNX27 aparecen en forma vesicular en células silenciadas para E6. Los niveles de Vps35 no se ven afectados por la presencia o ausencia de E6, sin embargo, se observa una recuperación en la co-localización de Vps35 con SNX27 posterior al silenciamiento de E6.

Los resultados sugieren que la interacción entre E6 y SNX27 está vinculada en las vías de reciclaje endosomal y proveen una función completamente nueva para las proteínas E6 de los VPH de alto riesgo.

DISCUSIÓN

La capacidad de las proteínas E6 de los VPH de alto riesgo para contribuir en el desarrollo del cáncer cervical requiere de múltiples funciones, dentro de estas funciones, está la de inducir la degradación de p53 al unirse con la proteína E6AP y promover su ubiquitinación. A pesar de los grandes avances en materia de investigación sobre los blancos celulares que interactúan y son degradados por E6, aún existe un gran desconocimiento sobre los procesos celulares que son alterados por el VPH durante el proceso infeccioso y carcinogénico. Una de las características exclusivas de los RNAm de los VPH de alto riesgo, es el procesamiento alternativo (*splicing*) que sufren, mediante el cual se generan formas truncas de la proteína, llamadas E6* (Smootkin *et al*., 1986). A pesar de que no se han atribuido capacidades inmortalizantes para estas formas truncas de E6, se han reportado ciertas actividades muy interesantes para estas proteínas, dentro de las cuales destaca la capacidad de la proteína E6* del VPH-16 para interactuar y modular la actividad de procaspasa 8 (Filipova *et al.,* 2007) junto con la forma completa de E6. Así mismo un análisis proteómico dio luz acerca de las proteínas que pudiesen ser blancos de las proteínas E6 de los virus de alto riesgo (Rozenblatt-Rossen *et al.,* 2012).

En este trabajo demostramos la capacidad de E6 de interactuar y modular las actividades de diferentes proteínas celulares.

Procaspasa 8

La capacidad de E6 de interactuar con procaspasa 8 está altamente conservada entre los distintos tipos de VPH. Mediante técnicas *in Vitro*, demostramos que los VPH de bajo riesgo (VPH-11) y los de alto riesgo (VPH-16 y VPH-18) así como las formas estrella poseen la capacidad de interactuar con procaspasa 8 y parece involucrar un mecanismo similar de reconocimiento, el cual esta mediado por el dominio efector de muerte. Además, detectamos que esta interacción es sensible a la temperatura, pues cuando los ensayos de interacción se llevaron a cabo a 4ºC, se observa una

disminución en la interacción, probablemente debido a los cambios conformacionales en las proteínas. Interesantemente, las proteínas E6 y E6* parecen interactuar más fuertemente con el DED que con la caspasa completa. Las razones por las cuales se observó este fenómeno son inciertas pero podría involucrar un cambio conformacional en el DED una vez que la caspasa es activada mediante su remoción, o bien de manera indirecta, ser un reflejo de la tendencia del DED a autoagregarse (Mielgo *et al*., 2009), y por lo tanto incrementar la cantidad de DED que forma complejos con E6, serán necesarios más estudios para demostrar esta cuestión. Por otra parte, la interacción de las formas E6* con procaspasa 8 no parece tener gran diferencia con respecto a las formas completas de E6, aunque pareciera que E6* de VPH-18 tiene un mayor potencial de asociación con procaspasa 8. Esta interacción parece estar modulada por la presencia de 12 aa en la porción C-terminal de E6*.

Una gran cantidad de blancos celulares son enviados a degradación por E6, sin embargo, no parece ser el caso de caspasa 8. Los resultados de degradación *in Vitro* indican que procaspasa 8 no es un blanco de degradación de E6 y que posiblemente esta interacción tenga un papel en el ciclo replicativo del virus. Por otro lado, caspasa 8 es una caspasa iniciadora en la cascada de señalización de apoptosis que finalmente lleva a muerte celular, debido a que E6 interactúa con procaspasa 8 y esta interacción aumenta los niveles de caspasa 8 activa, es lógico pensar que el proceso apoptótico se verá favorecido; sin embargo, este no es el caso pues los ensayos de anexina V demostraron que E6 no es capaz de alterar los niveles de apoptosis inducidos por caspasa 8 probablemente debido a que E6 induce una re-localización celular de caspasa 8 activa al núcleo.

La distribución de Caspasa 8 es primordialmente citoplásmica cuando es expresada en las células, pero, cuando es co-transfectada con E6, se observa una re-distribución de caspasa 8 al núcleo, lo cual también es observado en células HeLa. Posterior al silenciamiento de E6 en células HeLa se observa una disminución de la forma activa de caspasa 8, fracción p18, en el núcleo. Los análisis de inmunofluorescencia mostraron co-localización nuclear para ambas proteínas en células U2OS transfectadas con E6 y

Caspasa 8, de igual manera, en células HeLa se observa que la señal de caspasa 8 es nuclear y que, una vez silenciada la expresión de E6, hay un aumento en la señal citoplasmática de la caspasa 8. Nuestros resultados concuerdan con los reportados por Ocampo *et al*., (2008) en donde se observó la presencia de caspasa 8 en el núcleo de varias líneas celulares positivas a VPH, nosotros ahora demostramos que esto es debido en parte a la acción de E6. Estos resultados sugieren que la activación de caspasa 8 y su reclutamiento en el núcleo podrían ser esenciales en la ejecución de ciertas actividades importantes en el clico replicativo y/o mantenimiento del genoma, como lo establece Moody *et al*., (2009) en donde demuestran que la activación de caspasas es un paso crucial en la replicación del genoma viral, el cual está regulado por un corte dependiente de caspasas sobre E1 lo cual posiciona a E6 como un candidato en la regulación de este proceso.

FHL-2

La desregulación del factor transcripcional FHL-2, ha sido implicada en el establecimiento de distintos cánceres, sin embargo aún no es claro de que manera FHL-2 contribuye al proceso carcinogénico. Las actividades de FHL-2 están mediadas, en gran parte, por su distribución dentro de la célula, las formas citoplásmicas interactúan con integrinas y con la cinasa de adhesión focal y está involucrada en una gran variedad de transducción de señales mientras que las formas nucleares, interactúan con diversos factores de unión a DNA para controlar diversos procesos transcripcionales dentro de los que destacan AP1 y β-catenina. Altos niveles de estas proteínas han sido implicados con un aumento en la invasión tumoral y metástasis (Kleiber *et al*., 2007).

En el caso del cáncer de cérvix, aún no existen reportes acerca de su estatus de expresión en neoplasias asociadas a VPH. En el presente trabajo demostramos que las proteínas E6 de los VPH de alto riesgo son capaces de interactuar y modular los niveles de FHL-2. Mediante ensayos de interacción *in Vitro* se demostró la capacidad de E6 para interactuar con FHL-2 y que la expresión de E6, en células HeLa, induce un decremento en los niveles de la proteína . Una de las funciones de FHL-2 asociadas a

VPH es la modulación de la vía de Wnt al disminuir la actividad transcripcional de los genes blanco de esta vía por la oncoproteína E7 (Campo-Fernández *et al*., 2007). Es por ello que decidimos extender el análisis de interacción *in Vitro* y comparar las capacidades de las proteínas oncogénicas de interactuar con FHL-2. De manera sorpresiva, encontramos que E7 no es capaz de unirse a FHL-2 *in Vitro*, lo cual contrasta con los reportes anteriores.

Al demostrar que la interacción de FHL-2 es específica para E6 procedimos a evaluar el efecto biológico de dicha interacción. Un resultado interesante fue encontrar que FHL-2 no esta siendo degradado por E6 mediante E6AP y los niveles tan bajos de FHL-2 encontrados en células HeLa parecen involucrar otra ubiquitin ligasa, si embargo, cuando la expresión de E6 es abatida mediante siRNA encontramos una restauración en los niveles de FHL-2 en el citoplasma así como cuando la degradación proteasomal es inhibida, lo cual sugiere que E6 modula las actividades y localización de FHL-2 en la célula mediante el proteasoma pero sin involucrar a E6AP. Estos resultados identifican a FHL-2 como un nuevo blanco de interacción para las oncoproteínas E6 de VPH, e indican que el patrón de expresión perturbado podría ser un elemento importante en la carcinogénesis inducida por papiloma.

SNX27

La proteína E6 de los VPH de alto riesgo representan uno de los modelos más completos para la investigación en cáncer al ser una proteína que influye en muchos procesos celulares involucrados en la inmortalización y transformación celular. A la fecha continua siendo objeto de estudio con lo cual se han logrado entender algunos de los procesos celulares más importantes en el establecimiento de la carcinogénesis.

Dentro de los últimos estudios resalta el interactoma realizado por Rozenblatt-Rossen *et al*., (2012) en el que se identifica a SNX27 como nuevo blanco de interacción para E6. El presente trabajo demostró la capacidad de los VPH de alto riesgo de interactuar con SNX27 mediante el motivo de unión a dominios PDZ, siendo el residuo T156 es responsable de esta interacción. Así mismo se demostró que SNX27 y E6 co-localizan

en la células en un patrón vesicular, posicionando a la vía endocítica como una nueva vía que podría estar siendo modulada por E6. Si bien lo ensayos de degradación muestran que SNX27 no está siendo degradado por acción de E6 es importante recalcar que el patrón de expresión de SNX27 se ve alterado en células HeLa y que es restablecido una vez silenciado E6, aunque los niveles totales de SNX27 no se ven afectados cuando se llevo a cabo el western blot de proteínas totales probablemente por que E6 este implicada en interrumpir las funciones propias de SNX27 mediante impedimento estérico más que por su eliminación.

SNX27 es una proteína implicada en el proceso de maduración de endosomas hacia lisosomas, así mismo es una de las proteínas que regulan el tráfico celular, identificando proteínas en los endosomas dirigiendo el reciclaje hacia la membrana plasmática, principalmente por su interacción con componentes del Retrómero. E6 es capaz de evitar la interacción de SNX27 y Vps35 lo cual permite la posibilidad de que E6 este evitando la maduración de los endosomas, o bien, este regulando el proceso de reciclaje celular mediado por el Retrómero (Fig. 29).

Los resultados obtenidos dan luz acerca de nuevas funciones para las proteínas E6 de los VPH de alto riesgo, planteando la posibilidad de que E6 funcione como un regulador negativo en las funciones del Retrómero y reciclaje de ciertas proteínas. Son necesarios más análisis en el futuro para identificar los procesos celulares así como las proteínas que vinculan a E6 en las vía de reciclaje y elucidar el papel biológico de dichas interacciones.

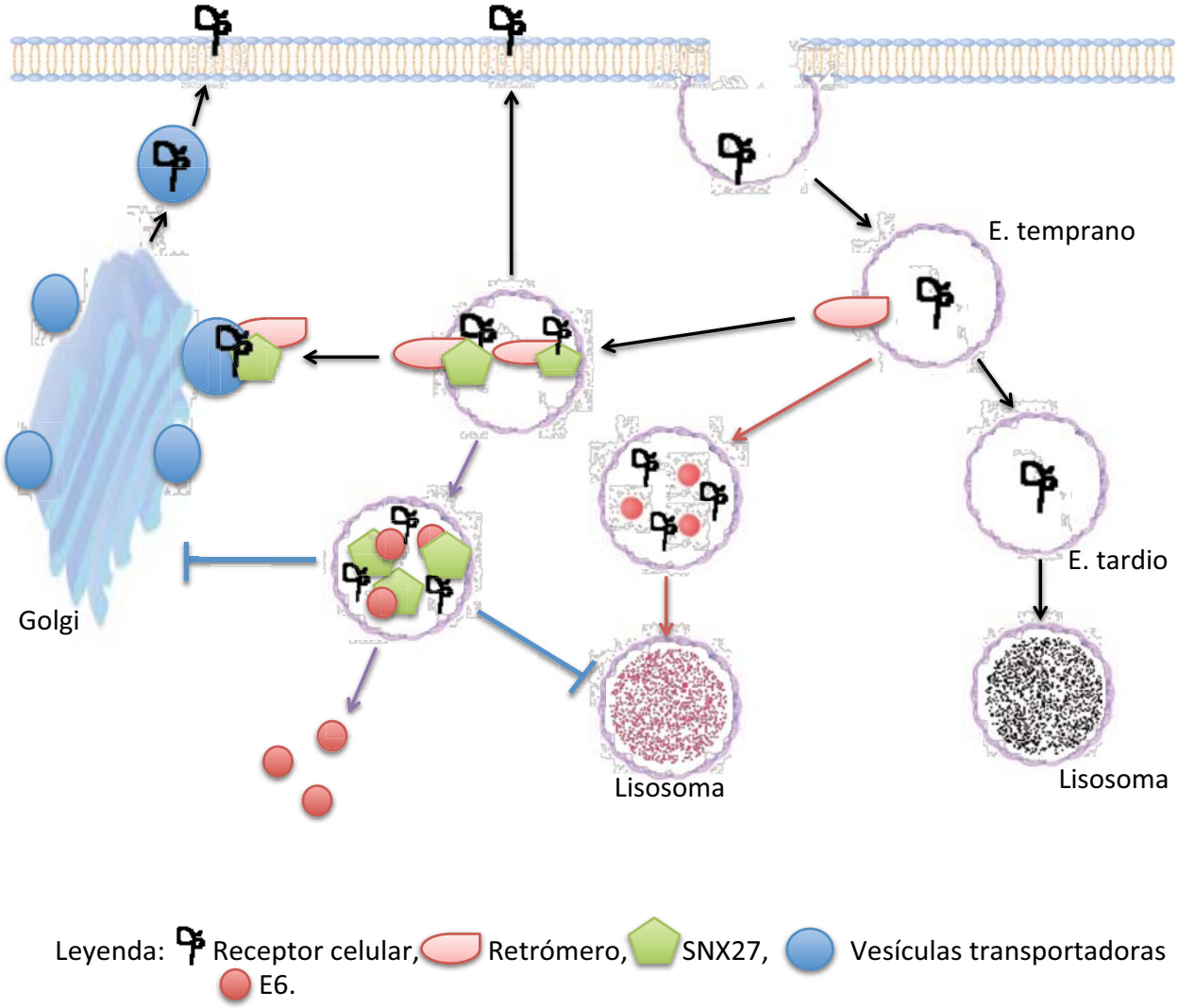


Figura 29. Posible función de E6 en vías de reciclaje celular. Los receptores de membrana normalmente son endocitados para su reciclaje, una vez formado el endosoma temprano, el receptor puede ser reciclado de manera inmediata o ser transportado a Golgi mediante su asociación con el Retrómero(y probablemente involucrando a SNX27); o bien, el receptor endocitado puede continuar el proceso de maduración a endosoma tardío y finalmente ser degradado por el lisosoma (**flechas negras**). Presumiblemente, E6 podría estar siendo atrapado en endosomas y posteriormente ser degradado por el lisosoma (**flechas rojas**). Sin embargo, en presencia de SNX27, E6 parece estar evitando su degradación lisosomal mediante la unión con SNX27 (**flechas moradas**), que a su vez, evita la interacción de SNX27 con el Retrómero conllevando al bloqueo en el reciclaje de receptores celulares (**flechas azules**).

CONCLUSIONES

Las proteínas E6 y E6* de distintos tipos de VPH son capaces de interactuar con la procaspasa 8 mediante el DED, al mismo tiempo que la activan e inducen su translocación a núcleo presumiblemente para iniciar el proceso de replicación del genoma viral.

Las proteínas E6 de los VPH de alto riesgo son capaces de interactuar con FHL-2 y modular la distribución celular de esta última. La regulación de los niveles de FLHL-2 son dependientes del proteasoma y no involucran a la proteína E6AP.

E6 de los VPH de alto riesgo interactúan con SNX27 mediante el dominio PDZ, la interacción comprende el aminoácido T156 sobre el PBM de E6. E6 modula la expresión de SNX27 en células HeLa y evita su interacción con proteínas miembro del Retrómero lo cual vincula a E6 en las vías de reciclaje celular.

Los resultados anteriormente expuestos demuestran la amplia gamma de procesos celulares alterados por la presencia de E6 y posicionan a esta proteína como un elemento clave en la comprensión del proceso carcinogénico inducido por VPH.

FUTURAS DIRECCIONES

Se propone estudiar la relevancia biológica de estas nuevas interacciones entre E6 y proteínas celulares en el ciclo de vida del virus, así como disectar el mecanismo molecular de su posible participación en el proceso carcinogénico. A su vez resulta de gran importancia estudiar la participación de otras proteínas de VPH en procesos de reciclaje celular y mantenimiento del genoma.

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ANEXOS

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The human papillomavirus (HPV) E6 oncoproteins promotes nuclear localization of active caspase 8

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ABSTRACT

The HPV-16 E6 and E6* proteins have been shown previously to be capable of regulating caspase 8 activity. We now show that the capacity of E6 to interact with caspase 8 is common to diverse HPV types, being also seen with HPV-11 E6, HPV-18 E6 and HPV-18 E6*. Unlike most E6-interacting partners, caspase 8 does not appear to be a major proteasomal target of E6, but instead E6 appears able to stimulate caspase 8 activation, without affecting the overall apoptotic activity. This would appear to be mediated in part by the ability of the HPV E6 oncoproteins to recruit active caspase 8 to the nucleus.

Introduction

Human papillomaviruses (HPVs) are the causative agents of cervical cancer (Bouvard et al., 2009). The virus encodes two oncoproteins, E6 and E7, which directly contribute towards the initiation of tumour formation and the maintenance of tumour development (Androphy et al., 1987; Banks et al., 1987; Smotkin and Wettstein, 1986). Loss of expression or inhibition of E6/E7 function in cells derived from cervical tumours results in a cessation of transformed cell growth and induction of either senescence or apoptosis (Butz et al., 1996, 2003; Jabbar et al., 2012; von Knebel Doeberitz et al., 1992). Therefore both E6 and E7 represent excellent targets for therapeutic intervention in HPV-associated malignancy. Both E6 and E7 exert their transforming activities through multiple mechanisms. In the case of E7, this involves inactivation of essential regulators of cell cycle control, of which the pRb family of pocket proteins are the most well-defined (McLaughlin-Drubin and Munger, 2009). The E6-induced inactivation of p53 and of PDZ domain-containing targets is also believed to play critical roles in the ability of the virus to induce transformed phenotypes (Banks et al., 2012; Moody and Laimins, 2010). However additional mechanisms are also likely to be involved, as evidenced by the broad array of novel interacting partners recently demonstrated in a variety of proteomic analyses of both the E6 and E7 oncoproteins from multiple HPV types (Rozenblatt-Rosen et al., 2012; White et al., 2012).

Although there are close to 200 different HPV types (zur Hausen, 2009), only a small subset of these, the so-called high-risk types, cause cervical cancer (Munoz et al., 2003). A great deal of effort has been expended in trying to identify the unique characteristics of these high-risk viruses that might be responsible for tumour development. One such potentially relevant feature of this group of viruses is the alternative splicing of the E6 ORF, which generates multiple short forms of the E6 proteins, termed E6* (Smotkin et al., 1989). In the case of HPV-18 E6*, only one such isoform is produced, whilst in the case of HPV-16 E6* at least three such isoforms have been reported (Doorbar et al., 1990; Roggenbuck et al., 1991). The functions of E6* are still a matter of intense debate. In some cases they appear to be able to negatively regulate the activity of the full-length E6 proteins (Pim and Banks, 1999; Pim et al., 1997), whilst in others they seem to have intrinsic functions of their own, and potentially modulate a variety of different signal transduction pathways (Pim et al., 2009). A series of recent studies provided indications that the HPV-16 E6 and E6* proteins can differentially affect caspase activity, an effect related to their ability to interact with caspase 8 and, in the case of full length E6, target it for degradation (Filippova et al., 2007; Tungteakkhun et al., 2010). These studies focused on the effects of E6 and E6* on cell survival and apoptosis induction pathways; however recent studies have also shown that caspase activation is an important aspect of the normal viral life cycle, appearing to be essential for the later stages of the viral life cycle in differentiating keratinocytes (Moody et al., 2007). We have

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therefore been interested in investigating how conserved was the ability of different HPV E6 proteins, and their alternatively spliced isoforms, to interact with and modulate caspase 8 activity.

Results

E6 from different HPV interact with pro-caspase 8 and DED in vitro

Previous studies had shown that HPV-16 E6*, and to a much lesser extent the full length HPV-16 E6, could interact with caspase 8 through the death effector domain (DED) (Tungteakkhun et al., 2010). Since modulation of caspase activation appears important for the viral life cycle (Moody et al., 2007), we were interested in determining whether the E6 proteins from other HPV types could also associate with caspase 8 in a similar manner. To do this, we performed a series of GST pull-down assays using purified GST fusion proteins of HPV-11 E6, HPV-16 E6, HPV-18 E6 and the two corresponding E6* proteins. Full-length caspase 8 was in vitro translated and radiolabelled with ^{35}S-methionine, and then incubated with the GST fusion proteins. Interaction assays were done at 4 °C, to reflect the conditions used in previous studies (Filippova et al., 2007), and also at room temperature. Following extensive washing the bound proteins were analyzed by SDS PAGE and autoradiography. The results in Fig. 1A demonstrate that all the E6 proteins analyzed showed a similar capacity to interact with the caspase 8, although HPV-18 E6* appeared to interact somewhat more strongly. Surprisingly, and in contrast to previous studies, we also observed association between HPV-11 E6 and caspase 8, although in all cases interaction was significantly stronger at room temperature than at 4 °C, demonstrating that the ability of E6 to bind to caspase 8 is cold sensitive. To extend these analyses to determine whether the site of interaction was mediated via the DED, we repeated the assays using the isolated in vitro translated radiolabeled caspase 8 DED. The results in Fig. 1B again demonstrate a similar level of interaction between the different E6 proteins and the DED, which is, again, cold sensitive. Again, high levels of interaction are seen with both the full length and E6* proteins, as well as with HPV-11 E6.

HPV-18 E6 interaction with DED is carboxy terminal domain dependent*

Previous studies had shown a significant difference in the ability of HPV-16 E6* and HPV-16 E6 to interact with caspase 8 (Filippova et al., 2007). In our analysis we noticed a slight increase in the capacity of HPV-18 E6* to interact with caspase 8 compared with the full length E6 protein. To investigate the mechanism by

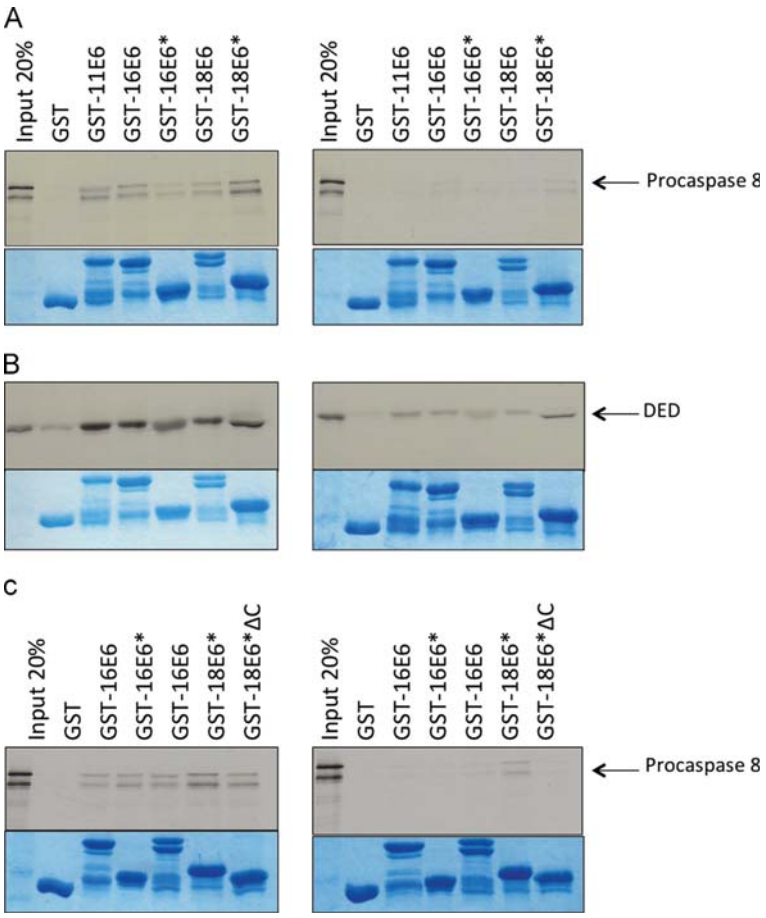

Fig. 1. Different HPV E6 proteins interact with the caspase 8 DED. The different HPV E6 fusion proteins were purified and incubated with *in vitro* translated and radiolabelled full-length caspase 8 (Panels A and C) or the isolated DED (Panel B) at either room temperature (left hand panels) or at 4 °C (right hand panels). After extensive washing the bound proteins were analyzed by SDS PAGE and autoradiography. Arrows highlight the bound caspase 8 or DED whilst the lower section in each Panel shows the Coomassie stained gel to show similar levels of GST fusion protein expression. The 20% inputs of the caspase 8 and DED are also shown.

which this could occur, we repeated the assay using wild type E6* plus a mutant where the carboxy terminal 8 unique amino acids were deleted. As can be seen from Fig. 1C, HPV-18 E6* does interact slightly more efficiently with caspase 8 compared with the full length E6 protein at both room temperature and 4 °C. Furthermore, this increased association would appear to require the amino acid residues lying within the unique carboxy terminal stretch of E6*. These results demonstrate that interaction between E6 and caspase 8 is conserved across both low- and high-risk HPV types, and, in the case of HPV-18 E6*, involves sequences that are unique to the carboxy terminal region of the protein.

E6 induces caspase 8 activation

In an extensive series of in vitro degradation assays, none of the above E6 proteins were found to have any effect upon the stability of caspase 8 (data not shown). Therefore we proceeded to investigate the effects of E6 upon the levels of caspase 8 expression in vivo. HEK293 cells were transfected with a caspase 8 expression plasmid, together with either HA-tagged HPV-18, HPV-16 or HPV-11 E6 expression plasmids. After 24 h the cells were harvested and the pattern of caspase 8 activation monitored by Western blotting using anti-caspase 8 antibody. As can be seen from Fig. 2A, the different E6 proteins slightly reduced the total levels of full-length (p57) caspase 8 expression. However, the amount of cleaved active (p18) caspase 8 increased significantly, in the presence of the full length

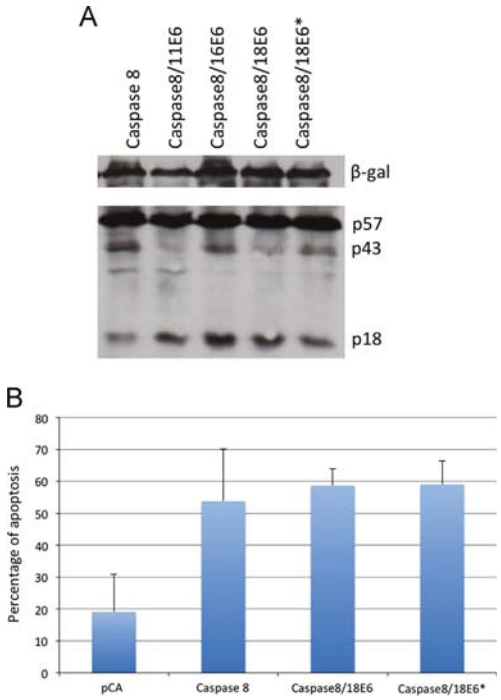

Fig. 2. HPV E6 proteins promote activation of caspase 8. Panel A. HEK293 cells were transfected with a caspase 8 expression plasmid either alone or together with the different E6 expression plasmids as indicated. After 24 h the cells were harvested and the levels of caspase 8 expression ascertained by Western blot analysis. Indicated is the full-length form of caspase 8 (p44) and the active cleaved form (p18). Also shown is the transfection and loading efficiency control of β-gal. Panel B. HEK293 cells were transfected with caspase 8 either alone or with HPV-18 E6 expression plasmids, as indicated, and after 24 h the cells were harvested and levels of apoptosis ascertained by Annexin V staining and FACS analysis. The results show percentage apoptosis from three separate experiments together with the standard deviations.

HPV-16 and HPV-18 E6 proteins, as well as with HPV-11 E6. In addition, a modest increase in caspase 8 activation was also observed with HPV-18 E6*. These results indicate that, although E6 does not significantly affect total caspase 8 levels in transient transfection experiments, it can nonetheless affect the degree to which caspase 8 is activated.

E6 binding to pro-caspase 8 does not induce apoptosis

Caspase 8 is the initiator caspase in the induction of the apoptotic cascade (McIlwain et al., 2013). Therefore we performed a series of assays to investigate whether the levels of increased caspase 8 activation seen in the presence of E6 was sufficient to increase the levels of apoptosis induced by caspase 8 overexpression. Cells were transfected with caspase 8, in the presence and absence of HPV-18 E6 and HPV-18 E6* expression plasmids, and after 24 h the levels of apoptosis were assessed by Annexin V binding and FACS analysis. As can be seen from Fig. 2B, E6 expression had a minimal effect on the levels of apoptosis induced by caspase 8, indicating that the increase in caspase 8 activation induced by E6 in Fig. 2A does not result in increased levels of apoptosis.

E6 induces caspase 8 nuclear translocation

Previous studies have shown that caspase activation is an important step in the HPV life cycle (Moody et al., 2007; Terenzi et al., 2008; Yu et al., 2007). Caspases are found primarily in the cytoplasm (Zhivotovsky et al., 1999) whilst E6 oncoproteins are expressed throughout the cell. We were therefore interested in determining whether E6 could in any way modulate the localization of caspase 8 within the cell. To do this, U2OS cells were transfected with a caspase 8 expression plasmid in the presence and absence of the HA-tagged HPV E6 expression plasmids. After 24 h, the cells were fixed and analyzed for the presence of HA-tagged E6 proteins and the co-transfected caspase 8 by immunofluorescence and microscopy. The results in Fig. 3 demonstrate that E6 has a diffused distribution, with both nuclear and cytoplasmic patterns of expression, similar to that reported previously (Guccione et al., 2004; Vaeteewoottacharn et al., 2005). Caspase 8, when transfected alone, is also predominantly found within the cytoplasm (Fig. 3A and Supplementary Fig. 1A). In contrast, when caspase 8 is co-expressed with either HPV-18 E6, HPV-16 E6 or HPV-11 E6 there is a significant degree of co-localization (Fig. 3B and Supplementary Fig. 1B and 1C) between E6 and caspase 8. In addition in cells where E6 nuclear localization is apparent, there is a corresponding increase in the levels of nuclear caspase 8 expression. Similar results are also obtained with HPV-18 E6*, where, again, there is a significant degree of co-localization with caspase 8, and a significant re-localization of caspase 8 to the nucleus. These results demonstrate that caspase 8 activation induced by HPV E6 is accompanied by an increase in the amount of caspase 8 found within the nucleus.

The presence of caspase 8 in the nucleus of HeLa cells is E6 dependent

In order to determine whether the pattern of caspase 8 was similarly affected by endogenous HPV-18 E6, HeLa cells were transfected with siRNA against luciferase as a control or against HPV-18E6/E7. After 72 h the cells were extracted into cytoplasmic, membrane, nuclear and cytoskeletal pools, and the pattern of caspase 8 expression ascertained by Western blotting. The results obtained are shown in Fig. 4A and demonstrate strong accumulation of p53 in the nucleus of cells transfected with siE6/E7. In the case of active caspase 8, there is a clear signal present within the

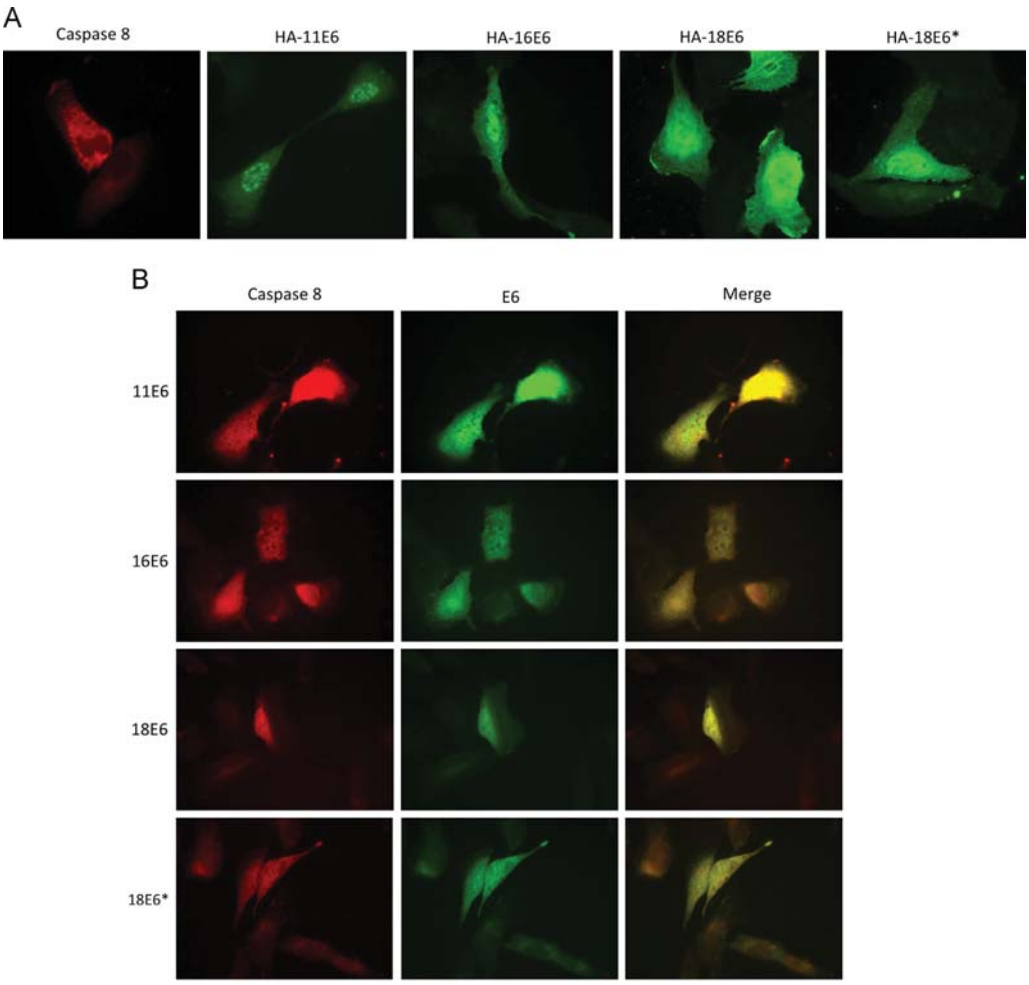

Fig. 3. HPV E6 modifies the subcellular distribution of caspase 8. U2OS cells were transfected with caspase 8 or the different E6 expression plasmids, either alone (Panel A) or in combination (Panel B) as indicated. After 24 h the cells were fixed and the patterns of caspase 8 expression ascertained by immunofluorescence analysis using anti-caspase 8 antibody and rhodamine-conjugated anti-mouse antibody (red), and HPV E6 detected by immunofluorescence analysis using anti-HA antibody and anti-rabbit Alexa 414 (green). Note the largely cytoplasmic distribution of caspase 8 and the nuclear/cytoplasmic distribution of the E6 proteins. Upon co-expression, there is marked co-localization of both proteins throughout the cell but an increase of caspase 8 in the nucleus in cells where E6 also displays nuclear accumulation.

nuclear fraction, and this decreases significantly upon siE6/E7 transfection.

We also performed immunofluorescence analysis of the patterns of caspase 8 expression in HeLa cells following siRNA ablation of E6/E7 expression. As can be seen from Fig. 4B, caspase 8 displays a predominantly cytoplasmic distribution in HPV negative HaCaT cells, whilst in control siRNA transfected HeLa cells caspase 8 has a significant proportion of nuclear localization. However, following transfection with siRNA E6/E7 there is a significant accumulation of cytoplasmic caspase 8. These results demonstrate that there are increased levels of nuclear caspase 8 in cells expressing HPV E6 oncoproteins.

Discussion

The ability of the high-risk HPV E6 oncoproteins to contribute towards the development of cervical cancer requires multiple functions, a number of which are likely to be absent from the low risk HPV E6 oncoproteins (Rozenblatt-Rosen et al., 2012; White et al., 2012). The capacity of high-risk HPV E6 oncoproteins to exist as an alternatively spliced shorter isoform termed E6* is one such unique feature of the high-risk HPV types (Doorbar et al., 1990; Smotkin et al., 1989). Although the E6* proteins do not appear to play a major role in the capacity of these HPV types to bring about cell immortalization or transformation (Sedman et al., 1991), they do nonetheless have a number of other intriguing activities. One of these is their ability, in conjunction with the full-length E6 proteins, to interact with and potentially modulate, the activity of caspase 8 (Filippova et al., 2007; Tungteakkhun et al., 2010). In the case of HPV-16, E6* appeared to enhance caspase 8-induced apoptosis, whilst the full-length E6 protein appeared capable of directing caspase 8 degradation. Thus, changes in the ratios of expression of the two forms of E6 could be expected to play a major role in viral pathogenesis. In the present study, we have found that interaction with caspase 8 is common to E6

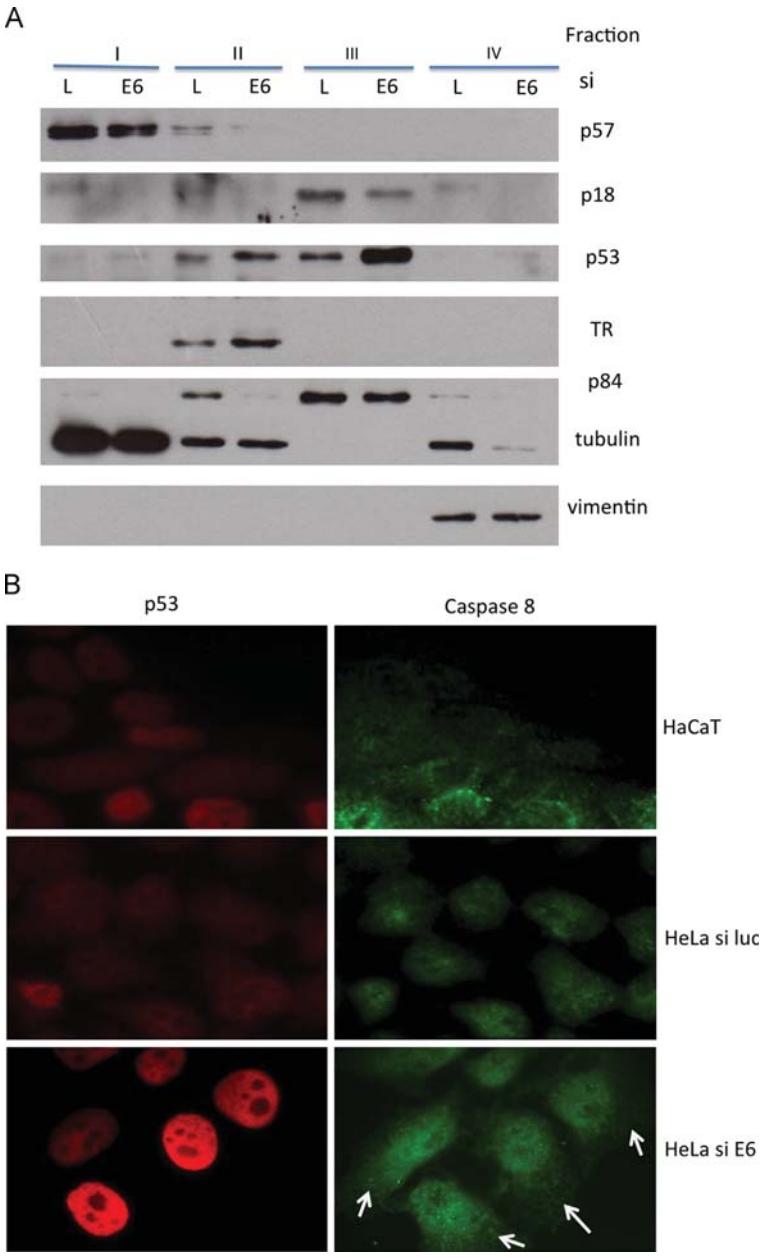

Fig. 4. HPV-18 E6 induces active caspase 8 expression in the nucleus of HeLa cells. Panel A. Cells were transfected with siRNA luciferase (L) or siRNA E6/E7 (E6) and after 72 h the cells were harvested and fractionated into cytoplasmic (I), membrane (II), nuclear (III) and cytoskeletal pools (IV). The fractions were then subjected to Western blot analysis for caspase 8 (p57 full length and active p18) and p53. Markers for loading and fraction integrity were vimentin, tubulin, p84 and transferrin receptor (TR). Panel B. HeLa cells were transfected with siRNA luciferase (si luc) or siRNA E6/E7 (si E6) and after 72 h cells were fixed and stained for p53 and caspase 8. HaCaT cells were included for comparison. Note the strong increase in nuclear p53 following E6/E7 ablation and the corresponding increase in cytoplasmic caspase 8, which is indicated by the arrows.

proteins of both high and low risk types, and that one consequence of this association is the stimulation of caspase 8 activity and concomitant re-localization to the nucleus.

The ability of different HPV E6 oncoproteins to interact with caspase 8 appears to be quite highly conserved. Using simple GST pull-down assays we found that high risk HPV-16 and HPV-18 E6 proteins, as well as low risk HPV-11 E6, could all interact with caspase 8. This interaction also seemed to involve a similar mode of recognition, with interaction appearing to be mediated via the caspase 8 DED. Interestingly, E6 proteins appear to interact more strongly with the isolated DED than with the full-length caspase 8. The reasons for this are unclear but could involve conformational changes in the DED once it is removed from the activated caspase, or be indirect and be a reflection of the tendency for an isolated DED to aggregate (Mielgo et al., 2009), and thereby increase the amount complexed with E6. Further studies will be required to

investigate these possibilities. Furthermore, in all cases the interaction between E6 and caspase 8 was cold sensitive, with only minimal levels of association detectable at 4 °C. Interestingly, we did not detect much difference between the abilities of HPV-16 E6 and HPV-16 E6* to interact with caspase 8, although HPV-18 E6* showed a modest but significantly increased potential to associate with caspase 8 compared with full length HPV-18 E6. Interestingly, this increased capacity to bind to caspase 8 appeared to be mediated through the unique carboxy terminal 8 amino acids of HPV-18 E6* protein.

A large number of HPV E6 substrates are directed for degradation at the 26S proteasome (Scheffner et al., 1993; for review see Banks et al., 2003), although in the case of caspase 8, E6 has little effect on the total levels of caspase 8 expression in cell. However the E6 proteins, and to a lesser extent E6*, all seem capable of stimulating caspase 8 cleavage to the active form of the enzyme. This would initially seem counterintuitive, as it might suggest that E6 was capable of stimulating caspase 8-driven apoptosis. However this does not appear to be the case, and this is most likely due in part to the ability of E6 to alter the subcellular distribution of caspase 8. Thus, caspase 8 when expressed alone is found primarily within the cytoplasm, whilst in the presence of E6 and E6* there is a marked increase in the amount of caspase 8 found in the nucleus. This occurs when E6 proteins are overexpressed and co-transfected with caspase 8, but is also observed with endogenous caspase 8 in HeLa cells, where there are high levels of nuclear caspase 8 which becomes much more cytoplasmic following siRNA ablation of E6/E7 expression. This nuclear accumulation of caspase 8 is in agreement with previous studies that reported increased levels of caspase 8 in the nucleus of HPV positive tumour cell lines and cervical cancers (Arechaga-Ocampo et al., 2008). Our results now indicate that this is most likely due to the activity of the E6 oncoprotein. These results suggest that E6 most likely recruits caspase 8 to the nucleus to perform functions that are beneficial either for the viral life cycle, or in the maintenance of cell proliferation in E6 transformed cells. What these activities are remains unclear, but could involve modulation of apoptotic pathways depending upon the levels of E6 expression (Filippova et al., 2007), or the targeting of substrates that would not normally be recognized by the caspase in these particular cellular locations. One such candidate would be the viral E1 protein, which is cleaved by caspases as part of the normal viral life cycle (Moody et al., 2007) and it will now be of interest to determine whether E6 has any potential role in this activity.

Materials and methods

Cells and transfections

Hek-293, HeLa, U2-OS and HaCaT cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were done by the calcium phosphate method or by FuGENE® HD Transfection Reagent (Promega) according to the manufacturer's instructions.

Plasmids

HA-tagged HPV E6 expression plasmids were described previously (Thomas et al., 2013). Pro-caspase 8 expression plasmid was obtained by cloning the pro-caspase 8 sequence into the pcDNA3 vector. The pCA-DED construct was obtained by cloning the coding sequence of Pro-caspase 8 death effector domain into the pCA backbone using standard PCR (BamHI and EcoRI sites). The products were verified by sequencing.

GST-fusion proteins purification

GST-fusion proteins production and purification were described previously (Pim et al., 1997).

In vitro translation and in vitro binding assays

An amount of 1 μg pro-caspase 8 or DED expressing plasmid was in vitro transcribed and translated using the TNT T7 Coupled Reticulocyte Lysate System® (Promega) employing [^{35}S]-methionine according to the manufacturer's instructions. For the *in vitro* binding assays equal amounts of the different E6 GST-fusion proteins were incubated with either *in vitro* translated pro-caspase 8 or *in vitro* translated DED in PBS at 4 degrees or room temperature for 90 min. After extensive washing with 0.2% NP-40/PBS the bound proteins were analyzed by SDS-PAGE and autoradiography. GST-fusion proteins amount was analyzed by Coomassie staining.

Immunofluorescence and cell imaging

About 24 h after transfection U2-OS cells were fixed in 3% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton-X100, the cells were then incubated for 2 h at 37 °C with polyclonal anti-HA probe (Santa Cruz biotechnology), monoclonal anti caspase 8 (1C12, Cell Signalling) either alone or in combination, washed extensively with PBS, and incubated with anti-mouse or anti-rabbit conjugated to fluorescein or rhodamine (Molecular Probes) at 37 °C. Slides were washed and mounted in Vectashield mounting medium (Vector Laboratories). HeLa and HaCaT cells were processed in the same manner 72 h post siRNA transfection and were incubated with anti p53 (Santa Cruz) and anti-caspase 8 antibody (IC12, Cell Signalling). Slides were analyzed with either a Leica DMLB fluorescence microscope with a Leica photo camera (A01M871016) or a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a 60× objective oil immersion lens.

siRNA silencing and fractionation experiments

HeLa cells were seeded in 6 cm diameter dishes and maintained for 24 h to allow attachment, then cells were transfected with siRNA (Dharmacon) against E6/E7 (5′CAUUUACCAG CCCGACGAG) and Luciferase as a control, using the Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. About 72 h after silencing, cells were harvested and protein extracted using the sub-cellular proteome extraction kit (Calbiochem) according to the manufacture's instructions, protein levels were evaluated by Western blot.

Western blotting and antibodies

After 24 h post transfection, cells were washed and directly lysed in sample loading buffer to generate a total cell extract which was analyzed by SDS-PAGE and Western blotting onto a 0.22 μm nitrocellulose membrane (Schleicher and Schuell). Membranes were blocked at 37 °C in 7% milk/0.1% Tween 20/PBS for 1 h, followed by incubation with the appropriate primary antibody diluted in 5% milk/0.1% Tween 20/PBS. After washing three times with 0.1% Tween 20/PBS membranes were incubated with HRP-conjugated anti mouse (DAKO) in 5% milk/0.1% Tween 20/PBS for 1 h. Blots were developed using Amersham ECL reagents according to manufacturer´s instructions. Primary antibodies were used as follows: anti-Caspase 8 (1C12) (Cell signalling) at 1:1000 which recognizes both the active and inactive forms of the

enzyme, anti-HA (Roche) at 1/1000 and anti-β-galactosidase (Promega) at 1/5000, anti p53 (Santa Cruz) at 1:1000.

Annexin V assay

HEK293 cells were seeded in a 6 cm diameter dish and transfected with a plasmid expressing Procaspase 8, either alone or in combination with HPV-18 E6 and E6* plasmids. After 24 h the cells were washed and trypsinized gently. They were then washed once in DMEM/10%FBS and once in PBS. The amount of apoptosis was then determined by incubating the cells with Annexin V-FITC using the Annexin-V-FLUOS Staining kit (Roche) according to the manufacturer's instructions. The cells were then analysed using a FACSCalibur fluorescence-activated cell sorter (Becton Dickinson).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.12.013.

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EXPERT OPINION

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HPV E6 oncoprotein as a potential therapeutic target in HPV related cancers

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Introduction: Human Papillomaviruses (HPVs) are the main etiological agents for the development of most ano-genital cancers and for a subset of head and neck neoplasias. The oncogenic capacity of HPV is due to the combined activity of the viral oncoproteins E6 and E7. A defining feature of all HPV associated cancers is the continued retention and expression of these two viral oncoproteins throughout the development of the disease, and this highlights their value as potential targets for therapeutic intervention, in HPV-induced malignancies.

Areas covered: In this review, the authors focus on the HPV E6 oncoprotein functions and its interactions with cellular targets containing either LxxLL motifs or PDZ domains. New approaches leading to the prevention such interactions are described, showing the advantage of E6 as a target for therapeutic intervention against malignant transformation and cancer.

Expert opinion: The high degree of conservation in E6–LxxLL interactions across multiple HPV types makes this a compelling therapeutic target for pathologies caused by diverse HPV types. Combining this with therapeutics directed against E6-PDZ interactions offers great promise for the treatment of malignancies caused by high-risk HPV types.

Keywords: cancer, E6, HPV, therapy

Expert Opin. Ther. Targets [Early Online]

1. Introduction

Human Papillomaviruses (HPVs) are small, non-enveloped, double stranded DNA viruses, belonging to the Papillomaviridae family. HPV has a circular double stranded DNA genome of about 8 Kb, encoding 8 open reading frames (ORFs) for viral proteins that are required for all aspects of the viral life cycle [1]. More than 150 HPV types have been identified, and 12 of these have been recently defined as cancer causing [2]. This separation allows a distinction to be made between virus types, with those associated with benign lesions being referred to as low risk (typified by HPV-6 and HPV-11) whilst cancer-causing types are referred to as high-risk (typified by HPV-16 and HPV-18). **Table 1** shows the list of HPV types that are cancer-causing, together with the associated anatomical site in which they are known to induce malignancy.

Papillomaviruses display a high degree of host and tissue specific tropism. They replicate exclusively in keratinocytes of the basal layer of the skin and mucosa, where they are believed to gain entrance to such cells through micro lesions in the epithelium [3]. Infection then begins with viral capsid attachment through the L1 capsid protein, to sulphated sugars on the surface of the exposed basement membrane [4,5]. Following attachment of the capsid to the as yet unidentified cell surface receptors, the virus enters the cell and becomes localised in endocytic compartments [6,7]. Following acidification of the endosomes, the viral capsid protein L2, in

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Article highlights.

- HPV-associated malignancies represent a major global health problem.
- The continued expression of the HPV E6 oncoprotein is necessary for maintaining tumour growth and represents an excellent target for the development of therapeutics for HPV-associated cancers.
- The abilities of E6 to interact with PDZ domains and LxxLL motifs play important roles in HPV-induced malignancy and the molecular details of such interactions are now well-understood.
- Therapies designed around inhibiting PDZ or LxxLL interactions of E6 are likely to be of great therapeutic value.

This box summarises key points contained in the article.

conjunction with the viral DNA, exits the late endosomes and enters the cell nucleus, most likely following nuclear envelope breakdown during mitosis [8,9]. The rest of the viral life cycle is then intimately linked to the differentiation of the infected keratinocyte, where there is coordinate expression of the different viral gene products, ultimately giving rise to new infectious viral particles (**Figure 1**). Thus, as the infected basal keratinocyte divides and the daughter cell begins to differentiate, the viral genome is also replicated at a low level, through the combined activity of the viral E1 and E2 proteins. These viral proteins are responsible for recruiting the cellular DNA replication machinery to the viral DNA origin of replication and for ensuring the correct segregation of viral genomes between the daughter cells [10,11]. As the cells differentiate E7 plays a major role in ensuring that these differentiating cells, which would normally have exited the cell cycle, actually remain in an S phase like state, and thereby allows the subsequent amplification of the viral genomes [12]. Under such conditions of unscheduled DNA replication the cells' normal response would be to enter an apoptotic programme. However in HPV infected cells this apoptotic response is blocked, largely through the activity of the viral E6 oncoprotein [13]. As the cells continue to differentiate in the upper layers of the infected epithelium, the late gene products, including E4 are produced [14,15]. Ultimately the two capsid proteins, L1 and L2, then self assemble and encapsidate the newly synthesised viral genomes and this thereby generates a new population of infectious virus particles [16]. This whole process normally takes around 2 – 3 weeks, but under certain circumstances these infections can persist for many months and years and are not cleared by the immune system. These long-term persistent infections are the single biggest risk factor for the ultimate progression to malignancy and for the development of cervical cancer [17].

During progression to malignancy there is a striking retention of expression of the viral E6 and E7 oncoproteins, which together drive the development of the tumour [18-22]. Indeed, in many cases the viral genome often becomes integrated into the host genome, and large sections of the viral genome are lost during this process. At this stage, the virus is no longer capable of replicating its DNA, and this can, therefore, be considered as a dead end as far as the viral life cycle is concerned [23,24]. However, this integration event ensures continued and uncontrolled expression of E6 and E7, which in turn drives the progressive changes that will ultimately result in the development of the cancer [25,26]. Indeed, numerous studies have shown that blocking the expression or the functions of either of these two viral oncoproteins will have a negative impact on the proliferation and development of the tumour. Most studies have been done on cells that have been grown in culture for many years, but even in this setting the cells are addicted to the continued expression of E6 and E7. Approaches taken include earlier studies using antisense RNA [27,28] or peptide aptamers to target E6 [29] or E7 [30]. More recently siRNA ablation of E6/E7 expression has proven to be been effective [31-33] as have RNA aptamers directed against E7 [34] and intrabody targeting of E6 [35]. In addition, use of proteasome inhibitors, which would be expected to block the activity of both E6 and E7 with respect to several of their target substrates have also been proposed as potential therapeutics for HPV induced malignancy [36,37]. More biologically relevant systems include mouse models of tumourigenesis, where loss of E7 expression, even in late stage tumours has been shown to result in tumour regression [21], and inhibition of E6/E7 expression in cells only recently explanted from tumour material also results in a cessation of cell proliferation [22]. In all of these cases, inhibition of E6 and/or E7 results in cell growth arrest/senescence and/or induction of apoptosis. Therefore, inhibiting the function of either protein is a valid therapeutic option for intervention in HPV-induced malignancy.

2. Why a therapy for HPV?

There are currently two HPV vaccines available, one that is bivalent and offers protection against HPV-16 and HPV-18 [38], whilst the other is quadrivalent and offers protection against HPV-6, 11, 16 and 18 [39]. Both vaccines appear to be extremely effective and without doubt will have a major impact on the global burden of HPV-associated malignancies [40,41]. However, it is important to remember that these vaccines are purely prophylactic, and have no therapeutic potential. Therefore, women who are already infected with the virus will still go on to develop cervical cancer, and it is likely to be up to 20 years before cancer-rates become affected by the current vaccines. Furthermore, the current vaccine requires a cold chain and is delivered over a 6 months period in 3 rounds of immunisation. In many parts of the world such levels of storage and patient retrieval are extremely difficult. Finally, there are also issues of vaccine uptake. Within the USA for example, uptake of the vaccine is often extremely variable and frequently very low [42]. Therefore, HPV-associated malignancy, unfortunately will remain a

Table 1. HPV associated cancers.

Site	HPV types	Attributable frequency	% of all cancer
Cervix	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and 73	100	4.5
Anogenital cancer other than those from the cervix			
Vulva	16, 18, 33	40	0.2
Vagina	16	50	0.2
Penis	16, 18	50	0.1
Anus	16, 18, 33	80	0.2
Cancers of the aero digestive tract			
Oral cavity	16, 18	23	0.1
Oropharynx and tonsil	16	35	0.1

HPV associated cancers and their frequency around the world [113,114].

pressing problem for many years to come and the development of effective therapeutics is as relevant today as it has always been.

3. Biological activities of the E6 oncoprotein

Whilst both E6 and E7 cooperate in the process of malignant transformation, they nonetheless have quite distinct biological activities that contribute to the development of the malignancy. Both proteins impact on a huge number of different cell regulatory pathways and in the case of E6 this is manifested in perturbation of a large number of different cell biological activities in the cell [43,44], which are summarised in **Figure 2**. As noted above, one of E6's major biological functions is inhibition of the cell's apoptotic responses, and this occurs through perturbation of the normal function of p53 and Bak [45,46]. In cooperation with E7, E6 plays an important role in the immortalisation of keratinocytes, and can also contribute to the generation of genome instability [47-50]. This in turn can contribute directly towards the development of the malignancy. A particularly intriguing activity of the high-risk HPV E6 oncoproteins is its ability to activate telomerase [51], which appears to correlate with its ability to induce both cell immortalisation and malignancy. In tissue culture systems, E6 also can induce many of the hallmarks of epithelial mesenchymal transition [52,53], and it can also perturb aspects of the immune response pathways [54,55]. Finally, in transgenic mouse models, E6 plays an important role in cooperating with E7 in tumour induction at diverse anatomical sites [56-59]. Of particular note, however, is the tendency for E6 not to play a major role during the early stages of tumour initiation, but a much more important role during tumour progression [60]. For this reason, E6 offers some particular advantages in being a target for therapeutic intervention during the later, more malignant stages of disease progression.

4. HPV E6 protein targets

Consistent with a protein exhibiting such diverse biological activity, HPV E6 can associate with a very large number of different cellular proteins and many of these are depicted in **Figure 2**. In such as review as this it would be impossible to discuss all of these in any great depth, however, it is important to highlight some of the targets that might offer some potential for the development of effective anti-viral therapeutics. Indeed, many of these associations are still relatively poorly characterised in terms of the molecular basis by which E6 interacts with the particular target protein. However, two classes of E6 protein partners appear to offer the most promise from a therapeutic point of view, both with respect to the associated biological activities and with respect to the detailed level of information available concerning the molecular basis for the protein recognition: these are substrates containing either LxxLL motifs or PDZ domains.

5. LxxLL Motif-containing targets

5.1 The interaction

A central facet of E6 function is its ability to interact with proteins that harbour LxxLL motifs [61-63]. The prototype partner bearing this motif, and arguably the most important is the ubiquitin ligase E6AP (E6-associated protein/UBE3A), although a number of other LxxLL motif containing targets of E6 have been described. E6AP was originally identified following studies demonstrating that E6 could direct the degradation of the cellular p53 tumour suppressor protein for ubiquitin-dependent proteasome-mediated degradation [64], an activity that was dependent on the recruitment of E6AP by E6 [65,66]. This activity appears to be a critical part of E6's ability to induce cancer [59]. Unlike many other tumours, p53 is invariably wild type in HPV-associated malignancies, which is consistent with E6's ability to target its degradation being viewed as analogous to mutational inactivation in other cancer types [67,68]. Furthermore, numerous studies in both tissue culture models and in transgenic animals indicate an important role for the ability of E6 to target p53, and potentially other substrates, through recruitment of the E6AP for both tumour development and induction of cell transformation [59].

Under normal circumstances, E6AP appears to play an important role in regulating the levels of expression of nuclear hormone receptors amongst other targets [69] and loss or mutation of the maternally acquired E6AP in the hypothalamus causes Angelman Syndrome, a severe neurological

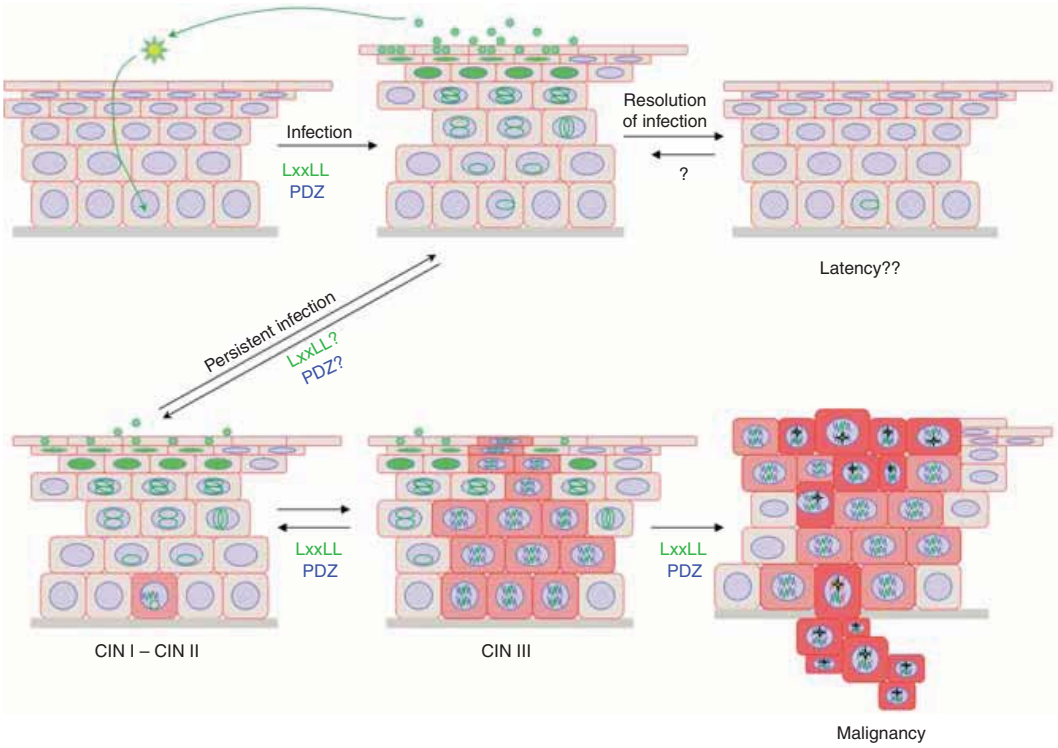

Figure 1. HPV life cycle and progression to malignancy. HPV is believed to infect the epithelial mucosa through micro-traumas in the skin. Following coordinate expression of its different viral gene products and amplification of the viral genomes during the differentiation of the epithelium this ultimately produces new infectious virus particles. Such lesions are often resolved and the virus is also believed to enter a poorly characterised latent state from which re-initiation of the life cycle may occur at a later date. Long-term persistent infection with the virus can result in the initiation of changes in the infected cells that will ultimately result in the development of malignancy. In addition, highlighted are the stages in this process where LxxLL and PDZ interactions are known to be critical. They are both essential for the normal viral life cycle, they may play roles in maintaining long-term persistent infections and they are intimately involved in the progression to malignancy. Currently, nothing is known about the potential involvement of LxxLL and PDZ interactions in HPV latent states.

developmental defect [70,71]. However, in HPV E6 expressing cells, the E6 protein associates very strongly with E6AP, which is then redirected by E6 to ubiquitinate, and thereby promote, the proteasome-mediated degradation of many of E6's target proteins [44]. However, this is not all that the interaction between E6 and E6AP achieves. Clearly, the ability of E6 to associate with E6AP is very important also for E6's own stability. Loss of association with E6AP causes a rapid loss of E6 protein through increased proteasome-mediated degradation [72]. Although the molecular basis underlying this observation is unclear, it nonetheless highlights the central importance of E6AP to a whole variety of different E6 functions. Recent studies have also made great strides in understanding the molecular basis by which E6 can interact with E6AP [73]. Crystallographic analysis of a recombinant HPV-16 E6 bound to a peptide containing the LxxLL motif (ELTLQELLGEER) shows that the two zinc-finger loops of E6, and the linker region between them form a deep pocket, into which fits the alpha helical LxxLL domain of E6AP. The three leucine (L^4, L^7, L^8) residues of this domain are aligned along the same face of the helix and insert into a hydrophobic pocket, while surrounding positive charges in the E6 pocket can bind the glutamic acid (E) residues of the peptide [73]. Other residues of the E6 pocket can interact with the other residues of the LxxLL region, and may contribute to the discrimination between different LxxLL-containing target proteins. Finally, an arginine residue in the C-terminal zinc finger of E6 locks this interaction in place, by interacting with the L^8 and with the E6 N-terminal zinc finger. In the absence of the LxxLL-containing ligand this binding cannot occur and the structure of E6 may be very different [73], which may also be one explanation for how interaction with E6AP might contribute to

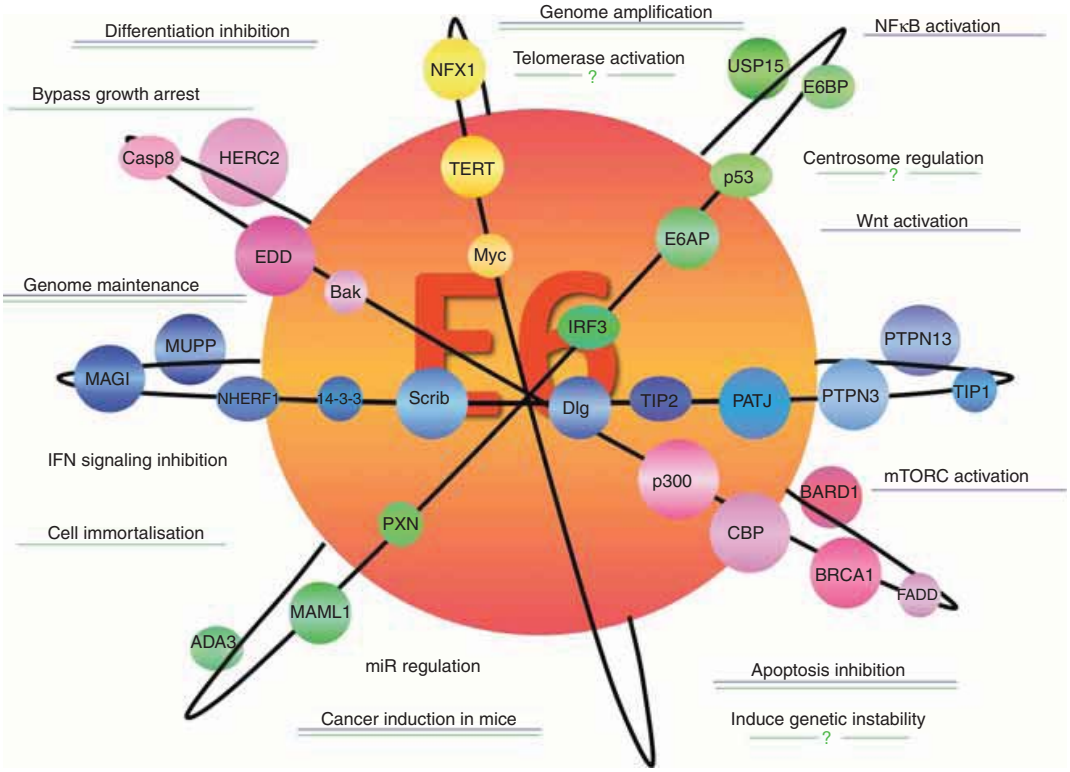

Figure 2. HPV E6 interacting partners and associated biological activities. E6 interacts with multiple cellular partners, through well-defined interaction motifs such as LxxLL (e.g., E6AP, MAML1 and PXN [shown in green]) or PDZ (such as Dlg, Scrib and MAGI [shown in blue]). Many other targets have more poorly defined mechanisms of interaction. Nonetheless E6 also perturbs a whole plethora of different cell functions, with those that are associated with LxxLL or PDZ interactions of E6 are underlined in green or blue, respectively.

stabilising E6. Analyses such as this offer unique opportunities for designing small molecule inhibitors that could be used to inhibit the association between E6 and E6AP.

5.2 Therapeutic consequences of blocking the E6–E6AP association

So assuming the interaction between E6 and E6AP could be inhibited, what would be the expected outcomes, both with respect to a normal viral infection and in the development of malignancy? One obvious consequence would be a stabilisation of many of E6's target proteins, whether this is p53 or other proteolytic substrates such as certain PDZ domain targets. Indeed, based on earlier structural studies and *in silico* screens, a number of lead compounds were identified that were potent inhibitors of the interaction between E6 and E6AP, and could rescue p53 from E6 induced degradation *in vivo* [74]. Similar results were also obtained using inhibitory peptides that could block association between E6 and E6AP [75]. Whether any of these compounds will be effective *in vivo*, however, remains to be determined.

As noted above, a critical element in the viral life cycle is the induction of DNA replication in cells that have entered terminal differentiation and the concomitant induction of a p53-dependent apoptotic response. Certainly, the ability of E6 to block this apoptotic cascade would be compromised if it were no longer able to degrade p53. Consistent with this, studies in raft culture models where E6 has been mutated such that it can no longer bind E6AP show significant defects in diverse aspects of the viral life cycle, with lower rates of late gene expression and viral DNA replication [76]. Likewise, such mutants exhibit some defects in their capacity to induce cell immortalisation, although with somewhat varying degrees [77,78]. In transgenic mouse models the ability of E6 to interact with E6AP appears to play a critical role in its ability to contribute towards the development of

malignancy [79,80]. At this stage, it is impossible to determine how many of these biological effects are due to lack of p53 degradation, or whether they are due to other E6AP-dependent activities of E6, such as targeting other substrates for degradation or maintaining E6's steady state levels in different biological settings. Indeed, some functions of E6 with respect to p53 inhibition, seem to be performed in the absence of degradation, apparently by blocking the ability of p53 to be acetylated by p300 and to activate gene expression [81]. Thus, blocking E6–E6AP association alone might still allow E6 to continue to perturb some aspects of p53 function. Finally, it is also worth noting that in cervical tumours, although p53 levels are low, p53 can often be readily detected by IHC [82-85]. This implies that in the context of tumour development *in vivo*, complete loss of p53 might not be such an overall requirement for malignant progression, certainly at certain specific stages of the malignancy.

Nonetheless, the elegant structural data, coupled with the important biological and biochemical consequences of the E6–E6AP association means that this interaction is a prime candidate for the development of inhibitory therapeutics.

6. PDZ domain targets

6.1 The interaction

The other well-defined class of E6 interactions occurs through PDZ (PSD95/Dlg/ZO-1) domain recognition. PDZ domains comprise stretches of amino acids of approximately 80 – 90 residues in length [86,87]. They are typically found as multiple repeats on proteins involved in assembling multiprotein signalling complexes, and are frequently found in proteins present at sites of cell–cell contact [88]. The ability of E6 to bind PDZ domains is dependent on the presence of a short stretch of amino acids at the extreme carboxy terminus of E6 [89,90], which constitutes a PDZ binding motif (PBM). Whilst LxxLL interactions of E6 appear to occur across the whole spectrum of different HPV types, regardless of the oncogenic potential of the virus, PDZ domain recognition is found only in the high-risk HPV types, where the PBM is found on cancer-causing HPV E6 oncoproteins but is absent from the E6 proteins of the low-risk virus types [91] and is depicted in **Figure 3**. This fact alone was an important driving force in the early studies on this class of E6 interactions since it suggested that the PBM of E6 might be a unique characteristic, which could contribute towards the development of malignancy. As can be seen from **Figure 3**, the PBM sequence is generally quite highly conserved across the different E6 oncoproteins from the different high-risk virus types, however, it is also worth noting that there are some significant differences. In addition, as can be seen from **Figure 2**, at least 12 different PDZ domain containing substrates of E6 have been described and an important open question is to try and understand which of these are relevant for E6's associated biological activities. The first targets to be described, Discs Large (Dlg) and Scribble (Scrib) both belong to a group of proteins that are linked to the regulation of cell polarity [89-93]. Indeed, early studies in *Drosophila* designated these as potential tumour suppressors [94], and more recent work on human tumours suggests that these may play similar roles, with loss of their expression being a common feature of diverse tumour types [95-98]. In fact, targeting cell polarity regulation appears to be a common mechanism whereby diverse human tumour viruses contribute towards the development of malignancy [99]. Particularly intriguing, however, are the differences in how the different HPV E6 oncoproteins target these various PDZ domain-containing substrates. In the case of Dlg and Scrib, each is targeted preferentially by different HPV E6 oncoproteins, where for example, HPV-18 E6 interacts preferentially with Dlg, whist HPV-16 E6 preferentially recognises Scrib [100]. This difference is all due to the L/V variation at the extreme carboxy terminus of E6; exchanging this last amino acid between the two E6 proteins also switches their substrate selectivity. Thus PDZ recognition is finely-tuned, and the structural studies that have been done from numerous laboratories, both biochemically and crystallographically, using recombinant PDZ domains and peptides containing the HPV-18 E6 C-terminal PBM ($RRRETQV^{-1}$), support this [101,102]. The core consensus type 1 PBM is shown in **Figure 3** (xS/TxV/L) and this is highly conserved over the E6 proteins from all the cancer-causing HPV types but is not found in low-risk types. PDZ domains are regions of 80 – 90 amino acid residues, comprising six beta sheets (βA-βF) and two alpha helices (αA and αB). The conserved folding of these elements forms a groove, with the carboxylate loop, or so-called GLGF motif, at the end. In fact, only the second G is completely conserved, and variation in the other residues of this motif can contribute to binding specificity [86,87].

It has been shown that under non-stringent conditions *in vitro*, the E6 PBM peptide can bind to PDZ domains that are not recognised *in vivo*; this is largely owing to the highly conserved nature of the PDZ domain and its ligand, where the baseline binding is provided by the S/T^{-3} and the V/L^{-1} residues of the PBM, which bind the αB helix and the carboxylate motif, respectively. This can be seen in the case of the PDZ3 of Dlg, which is not a target of E6 *in vivo*, but nonetheless can bind and form crystals *in vitro* [101]. In that analysis it was shown that 18E6's specificity of binding to the PDZ2 domain of Dlg is provided by the additional bonding of glutamine (Q^{-2}) to the carboxylate loop, and the arginine (R^{-5}) with the αB helix. The most strongly bound PDZ substrate of E6: MAGI-1 PDZ1, also binds in a similar manner, but with additional bonds being provided by the arginine (R^{-6}) binding to the βB/βC linker. Thus, the canonical S/T and V/L provide baseline binding, but specificity is provided by the non-canonical residues within the PBM and by the residues up to nine amino acid residues upstream [101-105].

The situation is also complicated by the fact that embedded within the PBM of E6 is a phospho-acceptor site on the S/T residue at the -2 position. Depending on the particular HPV

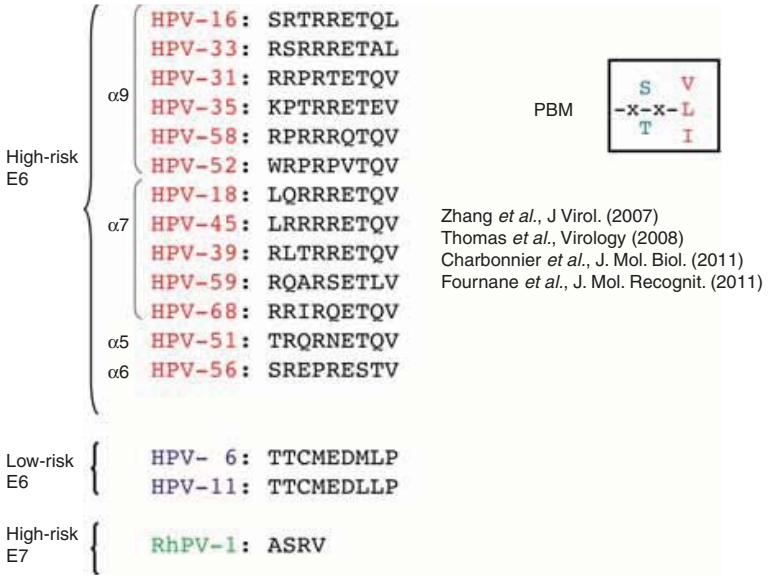

Figure 3. Sequence alignments of the carboxy terminal PBM of high-risk cancer causing HPV E6 oncoproteins. The figure shows the extended stretch of amino acids that are involved in PDZ recognition in comparison with the known core PBM consensus recognition sequence. Also shown are low-risk E6 oncoproteins and the carboxy terminus of Rhesus Papillomavirus E7, where the PBM is found on the E7 protein rather than on E6.

type, this residue can be phosphorylated by either AKT or PKA. In both cases, phosphorylation negatively regulates association with the PDZ domain containing substrate of E6, and instead confers binding of E6 to the members of the 14-3-3 family of proteins [106]. At present very little is known about the phospho-status of E6, either during the viral life cycle, or during the development of malignancy. However, studies *in vitro* indicate that the levels of E6 phosphorylation in monolayer culture are actually very low, suggesting that under these conditions PDZ recognition will be constitutive. Further studies are necessary, however, to determine how this might change *in vivo*, both with respect to any prognostic significance and potential impact on any PBM-PDZ blocking therapeutics.

As with the LxxLL containing partners of E6, the elegant structural and biochemical analyses that have been done to elucidate the molecular details underpinning E6-PDZ interactions makes this a compelling target for the development of novel inhibitory therapeutics.

6.2 Therapeutic consequences of blocking the E6 PBM

Whilst developing inhibitors that would block all E6-PBM recognition might be complicated by variations between the viral type and the identity of the PDZ domain containing substrate, the consequences of doing so would appear to offer varying degrees of benefit, depending on the nature of the lesion in question. In the context of the viral life cycle, mutations within the E6 PBM that prevent PDZ recognition, have marked deleterious effects on the ability of the virus to replicate and to promote genome amplification [107-109]. This activity appears to be mediated by the inability of these mutant viruses to stimulate epithelial hyperplasia, thus reducing the number of cells in the epithelium that are capable of replicating the viral genomes. However, in some cases, such mutant viruses are unable to maintain the viral DNA as an episomal plasmid, and high rates of viral integration into the host genome has been observed. Whilst this would undoubtedly be effective in clearing a viral infection, whether the corresponding enhanced likelihood of viral integration might not indirectly increase the risk of cancer development is an important factor to consider if any such therapeutics were to be developed.

Considering the ability of E6 to drive cell transformation and promote the development of malignancy, the inhibition of E6's ability to interact with its PDZ domain containing targets offers great promise. Numerous studies have shown that PDZ targeting is important for the ability of E6 to induce transformation in a variety of different tissue culture models [89,95,110]. However, exceptions nonetheless exist, where mutation of this region of E6 appears to have minimal effect [78]. These disparities most likely reflect a high degree of context dependency, and this is a recurring theme with many of the components of cell polarity control [99]. Likewise in animal models, blocking E6's ability to interact with its PDZ domain containing substrates would

be expected to block its ability to induce epithelial hyperplasia and to cooperate with E7 in the later stages of malignant progression [57,58,60]. However, variations are also observed depending on the precise anatomical site being assessed, again emphasising the context dependency.

7. Expert opinion

As noted above, LxxLL and PDZ targeting by E6 offer the best options for directed therapeutic intervention against E6 in HPV induced malignancy. However, neither target satisfies all the necessary criteria as the perfect intervention route for blocking all of E6's transformation related activities. Both play separate roles in the viral life cycle and in the development of malignancy, and loss of either set of interactions does not completely inhibit all of E6's biological activities. LxxLL targeting has many advantages, however, as the mode of association with E6 seems highly conserved across multiple HPV types, and any therapeutic would be expected to have a very broad range of activity. Indeed, many beta HPV types that have been implicated in the development of non-melanoma skin cancer, although not interacting with E6AP, nonetheless appear to associate by a very similar mechanism with MAML1, a protein involved in the regulation of Notch signalling in the skin [111,112]. Why the E6 proteins from mucosal HPV types interact preferentially with E6AP, whilst E6 proteins from cutaneous HPV types interact preferentially with MAML1, albeit through the same target sequences, remains an open question. However, any therapeutic that aimed to block E6-LxxLL recognition would be expected to have a very broad range of applicability. Conversely, therapeutic targeting of the E6–PDZ interaction might be undermined by the high degree of variation between the different HPV types and the post-translational modification to which the E6 protein is subject. Whether any agents that block E6-PBM recognition would also interfere with E6-14-3-3 recognition is still an open question and one that is worthy of further study.

Fortunately, there are few reports of sequence variation/mutation of E6 sequences from different geographical areas of the world. So whilst there are variants of E6 that appear to have increased potential to be linked with tumour development, none of these changes occur in regions that are involved in either PDZ or LxxLL interactions. Thus, any directed therapeutic would be unlikely to drive mutation of E6 and the development of resistant viruses.

Based on the above considerations, inhibiting LxxLL interactions would appear to offer the most potential for blocking E6 function, both in terms of blocking E6 substrate selection, broad specificity and perturbation of E6 stability. The precise mechanisms of action of any given inhibitor of E6-LxxLL interactions might, therefore, be different amongst the different HPV types, but it can be expected to have broad efficacy even if the molecular details underlying the observed phenotypes are not fully understood. As an adjunct to this approach, inhibition of E6 PDZ interactions might be something to consider in the later more malignant stages of disease development, where any potential drawbacks of blocking this activity of E6 are unlikely have any deleterious consequences. Obviously correct PBM–PDZ recognition plays an important role in cellular homeostasis and identifying compounds that specifically block E6–PDZ recognition whilst leaving normal cell PBM–PDZ interactions intact will be a difficult task.

In summary, suppression of E6 function using combinatorial approaches would appear to offer the best prospects for the development of therapeutics aimed at treating HPV-induced malignancy. Whilst major challenges remain in identifying and delivering such therapeutics, the wealth of biochemical and structural information that is now available about the mechanisms by which E6 targets many of its substrates now make this a much more feasible approach.

Declaration of interest

The authors declare that they have no competing interests. Lawrence Banks gratefully acknowledges research support provided by Associazione Italiana per la Ricerca sul Cancro, Telethon GGP10006 and the Wellcome Trust.

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The modulation of apoptosis by oncogenic viruses

Alma Mariana Fuentes-González, Adriana Contreras-Paredes, Joaquín Manzo-Merino and Marcela Lizano*

Abstract

Transforming viruses can change a normal cell into a cancer cell during their normal life cycle. Persistent infections with these viruses have been recognized to cause some types of cancer. These viruses have been implicated in the modulation of various biological processes, such as proliferation, differentiation and apoptosis. The study of infections caused by oncogenic viruses had helped in our understanding of several mechanisms that regulate cell growth, as well as the molecular alterations leading to cancer. Therefore, transforming viruses provide models of study that have enabled the advances in cancer research. Viruses with transforming abilities, include different members of the Human Papillomavirus (HPV) family, Hepatitis C virus (HCV), Human T-cell Leukemia virus (HTLV-1), Epstein Barr virus (EBV) and Kaposi's Sarcoma Herpesvirus (KSHV).

Apoptosis, or programmed cell death, is a tightly regulated process that plays an important role in development and homeostasis. Additionally, it functions as an antiviral defense mechanism. The deregulation of apoptosis has been implicated in the etiology of diverse diseases, including cancer. Oncogenic viruses employ different mechanisms to inhibit the apoptotic process, allowing the propagation of infected and damaged cells. During this process, some viral proteins are able to evade the immune system, while others can directly interact with the caspases involved in apoptotic signaling. In some instances, viral proteins can also promote apoptosis, which may be necessary for an accurate regulation of the initial stages of infection.

Keywords: Apoptosis, Virus, Cancer, Oncogene

Introduction

Various factors are associated with the development of cancer, including persistent viral infections, which are responsible of 15 to 20% of all neoplastic processes [1].

Studies related to infectious diseases and cancer have contributed significantly to our knowledge of cancer pathogenesis. Several Nobel prizes have been awarded to the researchers in this field [2], including Johannes Andreas Grib Fibiger (1926), for *Spiroptera* carcinoma and its association with gastric tumors in rats; Peyton Rous (1966), for cancer-inducing viruses; David Baltimore, Renato Dulbecco and Howard M. Temin (1975), for the interaction between tumor viruses and the genetic material of the cell; Michael J. Bishop and Harold E. Varmus (1989), for the cellular origin of retroviral oncogenes; and Barry J. Marshall and Robin J. Warren (2005), for the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease. In 2008 Harald zur Hausen shared the Nobel Prize award for his discovery of human papilloma viruses causing cervical cancer.

Other landmark studies have been of great relevance to the field. For example, in 1991, Harold zur Hausen proposed that a significant fraction of all human cancers worldwide, approximately 1 in 5, are associated with viral infections [3]. In 1910, Peyton Rous studied a cell-free transmissible oncogenic pathogen [4], and in 1932, Shope and Hurst demonstrated the oncogenic activity of a Papillomavirus in domestic rabbits [5]. In 1936, Bittner established the oncogenic role of mouse mammary virus [6], and in 1951, Gross confirmed the viral cause of murine leukemias [7]. In 1964, Epstein and collaborators showed the association of a virus with Burkitt lymphoma [8].

Many researchers have demonstrated the viral etiology of carcinomas of the uterine cervix. In 1974, Beral et al. proposed that cervical cancer was a sexually transmitted disease (STD) [9], and zur Hausen suggested that the

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Human Papillomavirus (HPV) was the putative oncovirus [10]. It is now indisputable that cervical cancer, penile cancer, some oropharyngeal cancers and other cancers of the anogenital tract are caused by certain strains of HPV. HPV vaccines have demonstrated effectiveness in reducing the incidence of cervical intraepithelial neoplasia [11], confirming the significant contributions of HPV to the development of cervical cancer.

During the same period, Vogel et al. presented preliminary data on the role of Hepatitis B virus (HBV) in liver cancer in Uganda [12], and in subsequent studies, a clear etiological link emerged between HBV and hepatocellular carcinoma [13]. This link was later extended to Hepatitis C virus (HCV) infections. In both cases, establish an association between the virus and tumor development has been complicated, by the long incubation period; the participation of chronic inflammation or cirrhosis in its pathogenesis; and the influence of cofactors, such as dietary and aflatoxins. The HBV vaccine, which was introduced in the last 15 years, has already demonstrated its potential for lowering the risk of hepatocellular carcinoma [14].

The effect of viral proteins in the modulation of cell proliferation and transformation has been widely studied [15,16], and it is now clear that oncogenic viruses may also interfere with the cellular control of apoptosis. Some oncogenic viruses have developed different mechanisms for evading apoptotic signals, mainly via the expression of viral oncogenes. During this process, the deregulation of the cell cycle and apoptotic pathways can lead to changes in the cell that eventually promote cancer development. Some of the mechanisms employed by oncogenic viruses to avoid apoptosis, thus promoting cell transformation, are provided in Table 1 [17-28].

In many instances, the regulation of apoptotic signaling has been associated with cancer development.

The study of the mechanisms by which viruses regulate apoptosis can contribute to the development of new therapies against infectious diseases and cancer. In this review, we will describe some of the mechanisms used by oncogenic viruses to modulate apoptosis.

Apoptosis

Apoptosis is a fundamental cellular process required for embryonic development, organogenesis and the elimination of damaged or aged cells during the maintenance of cellular homeostasis [29]. In the physiological context, apoptosis is strictly regulated. When this regulation fails, a number of pathologies may result, such as autoimmune or neurodegenerative diseases and cancer. Apoptosis is a form of cell death that involves a series of ordered events. The first phase is the commitment phase, wherein the cell loses contact with its neighboring cells and presents with modifications of the cytoskeleton, causing a decrease in cell size and changes in cell morphology [30]. During the second phase, the execution phase, there is an increase in intracellular Ca^{2+}, which induces the activation of certain groups of enzymes, such as endonucleases and proteases, such as caspases. Additionally, the chromatin is condensed and fragmented, forming vesicles of different sizes surrounded by a plasma membrane. These vesicles, known as apoptotic bodies, contain parts of the chromatin and cellular organelles [30,31]. The final phase is the termination phase, which involves phagocytosis and the degradation of the apoptotic bodies [30].

Apoptotic death is triggered by different intra- or extracellular stimuli. Intracellular death signals can be

Table 1 Human viruses related to cancer: viral proteins affecting apoptosis

Virus	Cancer type	Protein	Mechanism
Epstein Barr	Burkitt's lymphoma [17]	EBNA3C	Binds Rb and promotes cell cycle progression
	Hodgkin's lymphoma [18]	EBNA1	Inhibits p53 induced apoptosis
	Nasopharyngeal carcinoma [19]		
	Gastric carcinoma [20]		
Human Herpesvirus 8 (KSHV)	Kaposi's sarcoma [21,22]	LANA1 Kaposina	Bind to p53 and inhibit p53-dependent apoptosis
Human Papillomavirus	Cervical cancer [23]	E6	Inhibits p53, Bak, FaDD and procaspase 8
	Oropharyngeal carcinoma [24]	E7	Pleiotropic effects inhibiting and promoting apoptosis
	Anal cancer [25]	E2	Binds and activates caspase 8 (HPV-18);
			Interacts with c-Flip inhibiting its action
Human T-cell leukemia virus type 1 (HTLV)	Adult T-cell leukemia/lymphoma [26]	Tax	Involved in regulation of cell-cycle, apoptosis, cellular transcription, NFkβ, chromatin remodeling
Hepatitis B	Hepatocellular carcinoma [27]	HBx	Activates caspases 3 and 8
Hepatitis C	Hepatocellular carcinoma [28]	Core, NS3 and NS5A	Suppress p53-mediated apoptosis

induced by cell stress, which promotes the liberation of cytochrome c from the mitochondria [29]. Extracellular stimuli include UV radiation, the depletion of growth factors, and the ligand-mediated activation of death receptors.

The induction of apoptosis

Intrinsic and extrinsic pathways

In mammals, apoptosis is regulated by the activation of two signaling pathways: the extrinsic and the intrinsic pathways. The extrinsic pathway is regulated by membrane death receptors, such as DR4/TRAIL-R1 and DR5/TRAIL-R2. Tumor Necrosis Factor Receptor 1 (TNFR1), and Fas (CD95), are activated by their ligands TRAIL, TNF, and FasL, respectively. The binding of the ligand to its receptor induces the activation of the caspase cascade (Figure 1) [32].

Conversely, the intrinsic pathway is regulated by mitochondrial proteins, that upon activation, cause the release of cytochrome c into the cytoplasm [33,34]. In the cytosol, a complex known as the apoptosome is formed through the binding of Apoptotic Protease Activation Factor 1 (Apaf-1), procaspase 9, and cytochrome c [35-37]. The oligomerization of Apaf-1 activates caspase 9, which, in turn, induces the proteolytic cleavage of other substrates involved in cell death [33-35] (Figure 1).

At the biochemical level, when an inducer triggers a cell death signal in a target cell, the cell death process advances through enzymatic intermediaries, thus directing apoptosis. In both intrinsic and extrinsic pathways, the main effector proteins are the caspases [32,36].

The caspases constitute a family of cysteine proteases that are specific for aspartate. The caspase family members are similar in amino acid sequence, structure, and specificity [36]. Caspases are synthesized as zymogens, and their activation requires specific cleavage at selected aspartate residues. At the initial processing, an inactive caspase is cleaved in a large (p20) and a small (p10) subunits, after which the N-terminal domain is removed to form the catalytically active protease [32,36]. Caspases

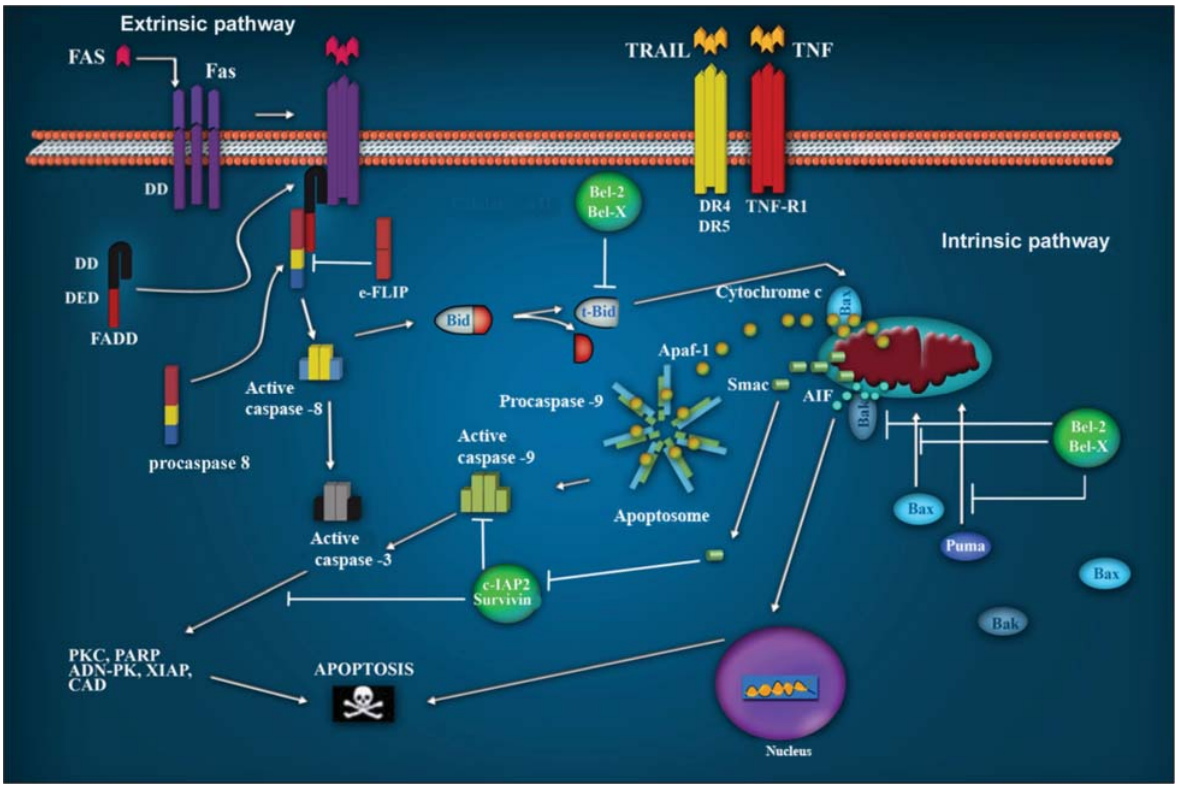

Figure 1 Apoptotic signaling pathways. The extrinsic pathway is regulated by membrane receptors. The interaction with their ligands Fas, Trail and TNF, favors their trimerization, inducing the recruitment of FADD through the interaction with their death domains (DDs). The interaction of FADD with procaspase 8 forms a complex called DISC, which favors its oligomerization and auto-cleavage. Active caspase 8 initiates the cascade of effector caspases 3, 6 and 7. In the intrinsic pathway, Bax and Puma are translocated from the cytosol to the mitochondrial membrane as a result of DNA damage, thus provoking the release of cytochrome c. Cytochrome c participates in the formation of the apoptosome, which is involved in DNA degradation. AIF contribute to DNA and nuclear fragmentation.

can be classified into two categories: initiator caspases and executioner caspases. Initiator caspases have a long N-terminal prodomain, which mediates the formation of protein complexes that provide the molecular platform for caspase activation and inhibition [32,36]. Initiator caspases cleave and activate a few specific substrates, including the zymogens of executioner caspases [32,36]. The activated executioner caspases then cleave their respective substrates, which elicit apoptotic cell death, along with its characteristic morphological features, such as membrane blebbing, pyknotic nuclei, cell rounding, and the formation of apoptotic vesicles [36].

Inhibitors of caspase activation (IAPs)

A balance between cell proliferation and apoptosis is required to avoid the development of pathologies such as neurodegenerative diseases and cancer. In eukaryotic cells, this balance is maintained mostly by a family of proteins known as IAPs (Inhibitor of apoptosis proteins) [37]. The IAP family is composed of 8 members; however, the best studied proteins in the family are the XIAP (X-linked inhibitor of apoptosis protein), that can directly inhibit the effector caspases (caspases 3 and 7) as well as the initiator caspase 9 [38]. Additionally, XIAP is an ubiquitin ligase; therefore it can indirectly inhibit apoptosis by inducing the degradation of caspases and other pro-apoptotic proteins via the proteasome [39].

Cancer cells express elevated levels of IAPs, which has been associated with chemoresistance, disease progression, and poor prognosis [40]. For example, under normal circumstances, survivin, a member of the IAPs that has been widely associated with the development of cancer, is only expressed in embryonic tissues; however, it has been found to be over-expressed in various tumors [41,42].

The role of oncogenic viruses in apoptosis

Although oncogenic viruses have been identified as etiologic agents in the development of different tumors, infection alone is not sufficient to induce cancer development. Most of the people infected by these viruses do not necessarily develop tumors. In those who do develop cancer, many years separate the initial infection and the appearance of a tumor, suggesting that many factors are involved in the transformation process.

A number of viral proteins that are responsible for the oncogenic capability of the virus, interact with elements of the apoptotic signaling pathways and thus inhibit their activities. Some viruses also regulate apoptosis by affecting its inhibitors, such as members of the IAP family and survivin. Conversely, other viral proteins can promote apoptosis, an event that is most likely important for the fine regulation of the initial stages of infection and is not necessarily involved in the transformation process.

Human papillomavirus

The main risk factor for the development of cervical cancer is the persistent infection of Human Papillomavirus [43]. Cervical cancer is the second most frequent cancer and the second leading cause of cancer death in women worldwide [44]. High risk HPVs (HR-HPV) refer to HPV types associated with cervical cancer, while Low risk HPVs (LR-HPV) are generally found in benign lesions or low grade cervical dysplasia [45,46].

Viral genome and structure

HPV is a small virus with a double-stranded DNA genome, that is organized into three distinct regions (Figure 2A). The early expression region (E) encodes proteins implicated in replication and the control of viral transcription (E1 and E2), as well as proteins that are involved in cellular transformation and immortalization (E5, E6 and E7) [47]. The late expression region (L) includes genes involved in capsid formation, L1 and L2. Finally, the region containing the binding sites for numerous factors that control transcription and viral replication is known as the Long Control Region, LCR or URR [48] Figure 2A.

Anti-apoptotic effect of HPV viral proteins

Many viruses, including HPV, have developed numerous strategies to block host-mediated apoptosis. The ability of HPV to persist in the host for long periods of time without being eliminated attests to the sophistication of its evasion mechanisms. A growing body of evidence suggests that the oncoproteins of HR-HPVs, E6, E7 and E5, can inhibit death receptor signaling at key points in the pathway (Figure 2B). In doing so, HPV is able to regulate the survival of infected cells to facilitate its replication cycle, thus ensuring the production and spread of its progeny [50]. HPV-positive cervical cancers and cell lines display a differential expression of several caspases and the downregulation of Fas expression, leading to impaired apoptosis [49,51]. Multiple alterations in both caspase expression and activation have been reported in biopsies and cervical cancer-derived cell lines that are HPV positive [49,51].

E7 protein

E7 oncoproteins from HR-HPVs can immortalize primary human keratinocytes. These oncoproteins inhibit differentiation and activate cell cycle progression, mainly due to the disruption of the pRb-E2F complex, releasing active E2F and trans-activating several genes involved in DNA synthesis [50]. In addition, E7 is a potent inhibitor of p21CIP1 and p27KIP1 activity, thus bypassing the normal G1 checkpoint control [52]. In addition to its role in cell proliferation and viral replication, E7 has pleiotropic effects on the cellular apoptotic pathways. It has been demonstrated that E7 from HPV-16 induces

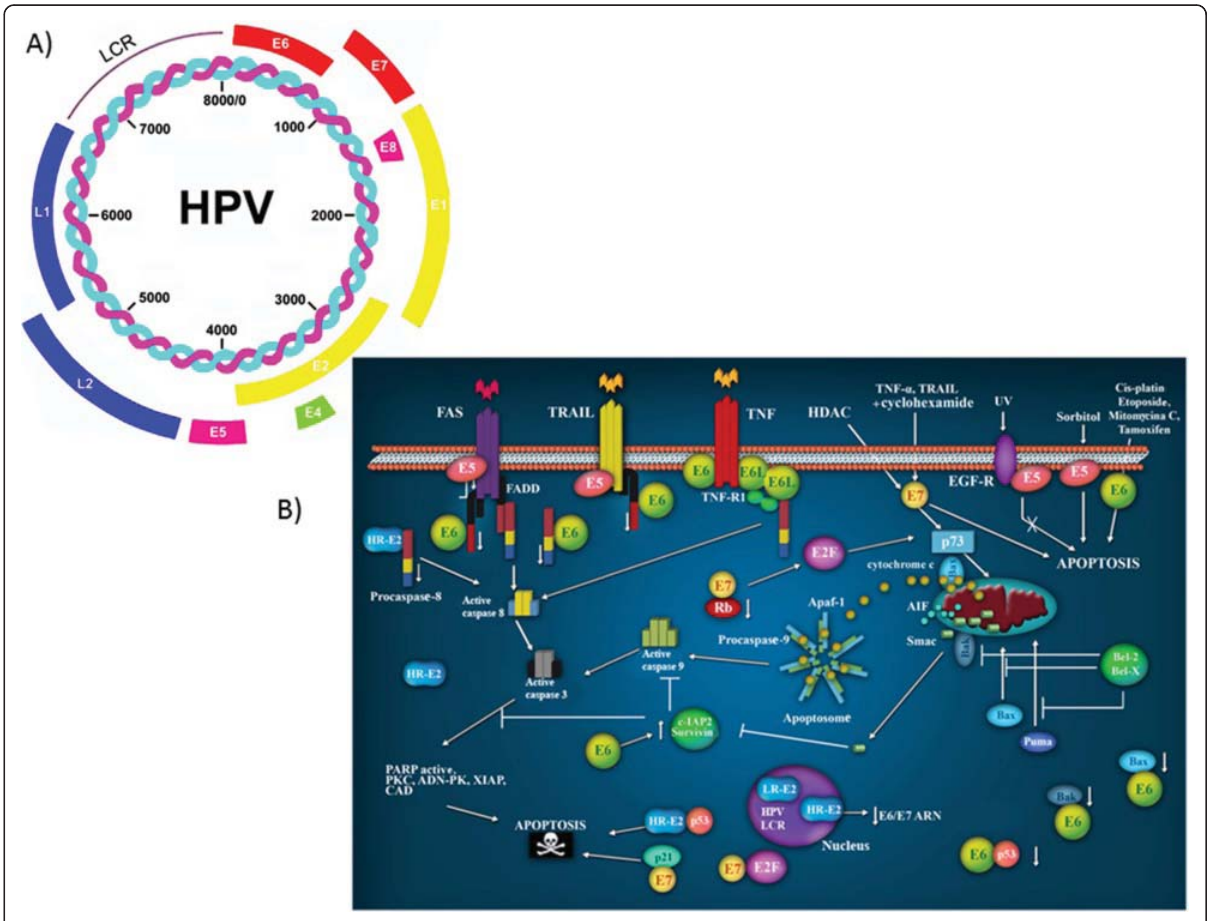

Figure 2 The HPV proteins involved in apoptotic signaling pathways. A) The Human Papillomavirus Virus genome. All HPVs have a common genomic organization and encode 8 proteins: E1, E2, E4, E5, E6 and E7 (early) and L1 and L2 (late). **B**) Participation of HPV proteins in apoptotic pathways. E5 impairs the formation of the death-inducing signaling complex triggered by FasL and TRAIL. E6 targets pro-apoptotic proteins such as p53, Bax and Bak for proteolytic degradation; in contrast, E6 can also induce the expression of IAPs. E7 promotes the degradation of the anti-apoptotic protein, pRb, releasing E2F-1. E2 induces apoptosis via the downregulation of E6/E7 mRNA; or direct binding and activation of procaspase 8; or when it binds to p53. Modified from Lagunas-Martínez A. et al. (2010) [49].

the degradation of pRb, an anti-apoptotic protein, through the ubiquitin proteasome pathway (Figure 2B) [52], suggesting that E7 might promote apoptosis. The majority of studies suggest that E7 has a pro-apoptotic role. It has been reported that when the HPV-16 E7 oncoprotein is expressed in the lens of transgenic mice, the cells are predisposed to undergo apoptosis that is both dependent on and independent of p53 [53]. Moreover, E7 has been shown to sensitize JD3 mouse lymphoma cells to IFN-alpha-induced apoptosis [54], the co-expression of E7 and p21 induces apoptosis in U2OS osteosarcoma cells [55], and the overexpression of E7 in genital-derived keratinocytes induces spontaneous cell death and sensitizes the cells to TNF-mediated apoptosis [56]. However, in some studies, E7 appears to be anti-apoptotic. Yuan *et al.* suggested that E7 can inhibit TNF-mediated apoptosis in keratinocytes by up-regulating the expression of the inhibitor of apoptosis protein, c-IAP2, and an antiapoptotic protein [57]. In another study, it was reported that the expression of E7 in fibroblasts delayed Fas-mediated apoptosis and prevented TNF-mediated apoptosis by suppressing caspase-8 activation [58].

The pleiotropic effects of both E6 and E7 on apoptosis is indicative of their important role in immune evasion and underscores the complexity of HPV-host interactions.

E6 protein

The E6 protein binds to numerous cellular targets implicated in proliferation and apoptosis. One of the functions of the HR-HPV E6 oncoproteins is the proteolytic inactivation of certain pro-apoptotic proteins such as p53 [59], Bak [60], FADD [61], procaspase-8 [62] and c-myc [63], through the ubiquitin proteasome pathway (Figure 2B).

Bak and myc were the first apoptosis-related targets of E6 to be identified. Thomas and Banks found that E6 inhibits Bak-mediated apoptosis by directly binding to Bak, an interaction that is conserved from HR- to LR-HPVs [64]. In laryngeal cells, E6 was found to inhibit TNF-mediated apoptosis by reducing the expression of Bak, without significantly affecting the expression of caspase-3 and caspase-8. As in the case with p53, both Bak and myc are ubiquitinated by E6AP, are able to bind to E6 and are degraded in the ubiquitin-proteasome pathway [64].

E5 protein

Recent studies have shown that the E5 protein inhibits apoptosis mediated by the TRAIL and Fas receptors (Figure 2B). E5 reduces the affinity of Fas for its ligand. It blocks the TRAIL-mediated apoptotic signaling pathway by preventing the formation of the TRAIL-DISC complex and inhibits the proteolysis of caspases 8 and 3, as well as of PARP [65].

E5 also protects tumor cells from apoptosis induced by UV-irradiation by enhancing the PI3K–Akt and ERK1/2 MAP kinase signaling pathways [66]. In addition, the HPV-16 E5 protein inhibits hydrogen peroxide-induced apoptosis by stimulating the proteosomal degradation of Bax. In contrast, E5 was also reported to sensitize human keratinocytes to apoptosis induced by osmotic stress [66]. However, this effect may be due to cell membrane modifications caused by the highly hydrophobic E5 protein. By modulating apoptosis, HPV 16 E5 allows HPV 16-infected cervical cells to evade apoptosis induced by physical or chemical stimuli. In addition, HPV 16 E5 may protect infected cells from apoptotic stimuli derived from immune effector cells by impairing FasL- and TRAIL-mediated apoptosis, thus contributing to the evasion of host immunosurveillance. All these activities may ultimately lead to cervical carcinogenesis.

The pro-apoptotic effect of viral proteins

Viral infections can also promote pro-apoptotic processes, and these opposing effects on apoptosis can be mediated by the same proteins. For example, E6 and E7, which can inhibit apoptosis, can also promote it. The viral apoptotic effect is better understood during the establishment of an infection. The life cycle of HR-HPV involves the fine regulation of the expression of viral oncogenes that will allow the cellular differentiation necessary to produce viral particles.

Moody *et al*. [67] reported that HPV proteins activate rather than suppress caspases, and this could be a necessary condition for the productive HPV life cycle. The authors observed that the treatment of HPV-31-positive cells with caspase inhibitors significantly reduced viral genome amplification. The identification of a caspase 3/7 cleavage site (^{46} DXXD ^{49}) in the viral replication protein E1, which is conserved in all genital HPVs, suggests that this motif provides an important function in the differentiation-dependent life cycle of papillomaviruses [67]. It is possible that the expression of antiapoptotic proteins, coupled with a low level of caspase activation, may be important in providing the balance between cell viability and cell death upon differentiation.

Protein E2

The viral E2 protein plays a critical role in the HPV life cycle due to its ability to regulate viral DNA replication and the transcription of E6 and E7 oncogenes [68]. The integration of the viral DNA into the cellular genome is considered a key element in the transformation process. Viral episome rupture during integration frequently occurs in a zone that limits E2 expression. Therefore, it is probable that the effects of the full-length E2 will occur preferentially during the initial stages of infection.

The direct induction of apoptosis by E2, independently of E6 and E7, was first demonstrated in 1997 by Frattini *et al*. [69], who observed the death of human foreskin keratinocytes, when they were infected with adenovirus expressing E2 from HPV31. Desaintes *et al*. [70], showed that in HeLa cells, apoptosis was induced only by the full-length E2 protein from HPV18, and not when the transactivation domain of E2 was deleted. As both proteins can repress the transcription of E6 and E7, this result indicated that apoptosis does not occur through the repression of the viral oncogenes.

Some studies have shown that E2 can induce apoptosis in HPV negative cell lines. Furthermore, this protein binds to and activates pro caspase 8, through its transactivation domain, overcoming the need for adaptor proteins involved in the classical extrinsic pathway that is Fas-dependent [71] (Figure 2B).

The involvement of caspase 8 in apoptosis induced by E2 was also demonstrated in HPV16, in which E2 directly interacts with c-FLIP [72].

Because E2 is expressed in the intermediate differentiated layers of the HPV infected lesions, it is possible that in vivo, the modulation of caspase 8 by E2 might play a role in the formation of warts, via an as yet unknown mechanism [73].

The role of p53 in E2- induced apoptosis is controversial. E2 induces apoptosis in HPV positive- and negative-cell lines through both p53 dependent and p53 independent mechanisms [71,74]. It is worth mentioning that E2 proteins from the low-risk HPV6 and HPV11 cannot induce apoptosis, which could be due to their cellular localization, because the E2 proteins of low-risk HPVs are located only in the nucleus, whereas those of HR-HPVs are localized in the both nucleus and cytoplasm [74].

Even the role of E2 in apoptotic induction in HPV life cycle is not yet understood, this effect could be related to the activation of E1 during viral genome replication. E2 could also be inducing apoptosis in those cells that do not allow the virus to properly complete the viral cycle.

Hepatitis viruses

Liver cancer or hepatocellular carcinoma (HCC) is the third leading cause of cancer related deaths in the world [75]. It is the fifth most common cancer in men and the eighth in women. The Hepatitis virus is the main etiologic agent of HCC [76]. The Hepatitis viruses are the most common infections that affect the liver. To date, 5 responsible agents for hepatitis have been identified and are characterized as follows: Hepatitis A virus (HAV), B (HBV), C (HCV), D (HDV), and Hepatitis E virus (HEV). HBV and HCV are responsible for 70% of hepatocellular carcinoma, of which 60% are caused by HCV [77,78]. This phenomenon can be explained by certain biological and clinical characteristics of HCV that favor hepatocarcinogenesis, such as the high capacity of HCV to induce a chronic infection. In contrast, after 10 years of infection, HBV only induces chronic cirrhosis in a small percentage of patients (5-10%), while the percentage of patients who develop this disease as a consequence of HCV infection is 55-60% [79].

Viral genome and structure

HCV belongs to the *Flaviviridae* family [80]. Its genome comprises a single strand of DNA which encodes a single 3000 bp open reading frame (ORF), flanked by untranslated regions (UTR) at the 5′ and 3′ ends (Figure 3A) [81]. The ORF encodes a polyprotein that is processed to produce three structural proteins, core C, E1 and E2, a small integral protein p7, and six nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The structural proteins are found in the N-terminal region, while the nonstructural proteins are encoded by the C-terminus (Figure 3A) [82]. The main functions of these viral proteins are summarized in Table 2 [83-91] Figure 3A.

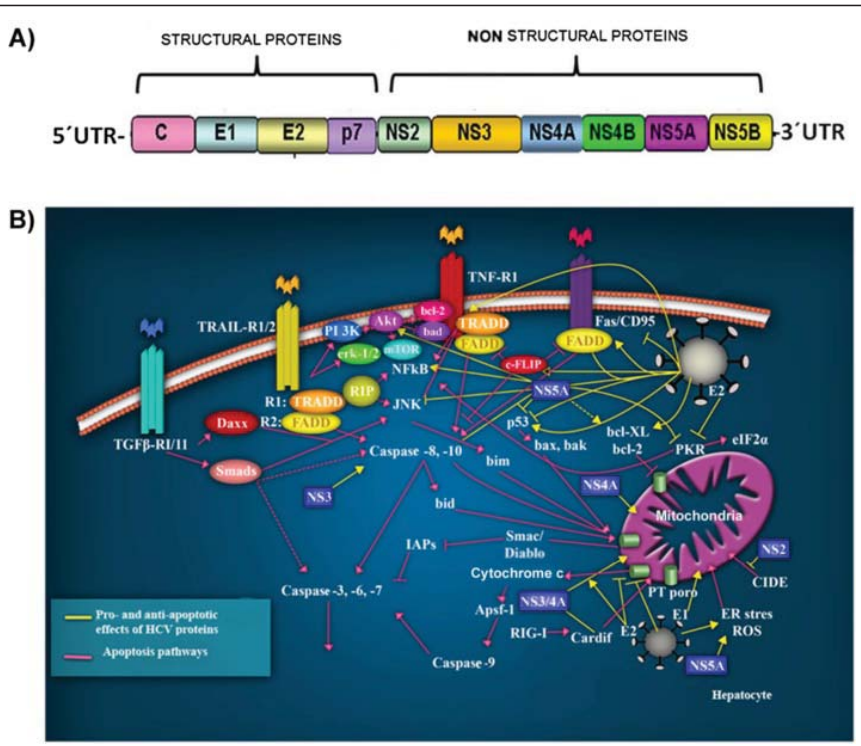

Figure 3 The Hepatitis C virus and apoptotic signaling pathways. A) The Hepatitis C virus genome. A single open reading frame encodes four structural proteins and six nonstructural proteins. **B**) HCV-infected hepatocytes are recognized by the immune cells, that promote apoptosis via the death receptor ligands, TRAIL, TNFα, CD95 ligand, and TGF-β, as well as granzyme B/perforin (**Pink lines**). Ligand-induced apoptosis activates caspase-8, whereas activation of caspase-9 occurs via the mitochondrial permeability transition (PT) pore, triggering the activation of caspases cascade and the irreversible induction of apoptosis. For virtually all HCV proteins, pro- and anti-apoptotic effects have been described (**Yellow lines**). The structural (core C, E1 and E2) and nonstructural (NS2, NS3, NS4A and NS5A) proteins participate in the extrinsic and intrinsic apoptotic pathways. Modified from Fischer R. et al. (2007) [92].

Table 2 Functions of HCV viral proteins

Structural proteins	Functions
Core	Forms the nucleocapsid and participates in signaling pathways that control cellular proliferation and apoptosis [83].
E1 and E2	Glycoproteins that make up the viral envelope, they bind to the receptor on the host cell to facilitate viral entry [84].
P7	Creates hydrophobic pores with ionic channel activity. Fundamental in the assembly and release of viral particles [85].
Nonstructural proteins	
NS2	Forms a catalytic complex with NS3 to cleave the NS2-NS3 junction. Important for the production of infectious viruses [86].
NS3	Serine protease that cleaves the junctions between NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. Contains a helicase domain implicated in viral replication. Cofactor NS4A [87].
NS4A	Important cofactor for NS3 activity participates in host innate immune response evasion and regulates viral transcription [88].
NS4B	Induces formation of membranous web specialized sites where RNA replication takes place [89].
NS5A	Binds viral RNA participating in viral replication; promotes particle assembly [90].
NS5B	Is the RNA polymerase responsible for replication of the viral genome [91].

Apoptotic processes induced by HCV infection

The induction of apoptosis is a mechanism used by hepatocytes to defend against HCV infection. The immune response is mediated mainly by macrophages and natural killer (NK) cells, which can directly cause the death of the infected cells [93]. Additionally, this process can be mediated by the receptors and ligands of the Tumor Necrosis Factor family, specifically, the TNFα/1 receptors, CD95/CD95 ligands, and TRAIL receptors 1 and 2 (Figure 3B) [93]. The binding of the ligands to the death receptors results in the activation of caspase 8 which in turn, activates two signaling pathways. The first pathway involves the proteolytic cleavage of Bid, the release of mitochondrial cytochrome c, the activation of caspase 9 [94], and the effector caspases 3, 6 and 7. In the second signaling pathway, caspase 8 directly activates the effector caspases. In this case, apoptosis is also regulated by inhibitors, such as survivin and c-FLIP, which can block caspase activity [95]. HCV viral proteins have the ability to inhibit host induced apoptosis, fact that could allow the establishment of a persistent infection.

The core protein

It has been demonstrated that the core protein of HCV has both pro-apoptotic and anti-apoptotic functions. This protein can inhibit CD95 receptors and TNFα induced apoptosis by inhibiting the liberation of cytochrome c and, thus, by activating caspases 9, 3 and 7 (Figure 3B) [96]. Additionally, the direct binding of the core protein to the cytoplasmic domains of the CD95 and TNFα receptors has been reported to induce a pro-apoptotic effect by altering mitochondrial function. Specifically, this effect induces the production of reactive oxygen species, causing a change in mitochondrial membrane potential, which permits the release of cytochrome c [97]. Furthermore, it has been postulated that this protein can bind to death domains, such as FADD and to the c-FLIP inhibitor, resulting in an anti-apoptotic effect [98]. Many studies have indicated that the core protein can modulate p53 in a positive or negative manner [99,100].

HCV can also induce apoptosis through the interaction of NS5A with the protein kinase R (PKR), the kinase regulated by double-stranded RNA (dsRNA). PKR has different functions, such as the evasion of the antiviral action of interferon and the induction of apoptosis. This kinase catalyzes the phosphorylation of the transcription factor eIF-2, leading to the inhibition of anti-apoptotic protein synthesis during viral infection [101] (Figure 3B). In turn, PKR is activated via binding to the NS5A viral protein.

E1 and E2 proteins

As is the case for other oncogenic viruses, is clear that Hepatitis C has a dual role in regulating apoptosis. For instance, HCV E1 and E2 proteins, which mediate the binding and entry of HCV into the host cell, are capable of inhibiting Fas-mediated apoptosis by repressing the activation of caspase-8 and the release of cytochrome c from the mitochondria [102]. However, these structural proteins increase the expression of FasL and the ability of hepatocytes to induce apoptosis in activated CD4+ and CD8+ T cells, which may contribute to the persistence of HCV [103,104].

Nonstructural proteins

Figure 4 shows the roles played by HCV nonstructural proteins in the apoptotic pathways. The processing of nonstructural proteins involves the formation of autocatalytic protein complexes. NS2 is a transmembrane protein, found in the endoplasmic reticulum. It binds to and activates cell death-inducing DNA fragmentation factor (DFFA)-like effector b, (CIDE-B), which is a key inducer of the extrinsic apoptotic pathway [105].

The NS3 protein promotes the degradation of Cardif, a protein that translocates to the mitochondrial membrane and activates the intrinsic pathway [106]. When it

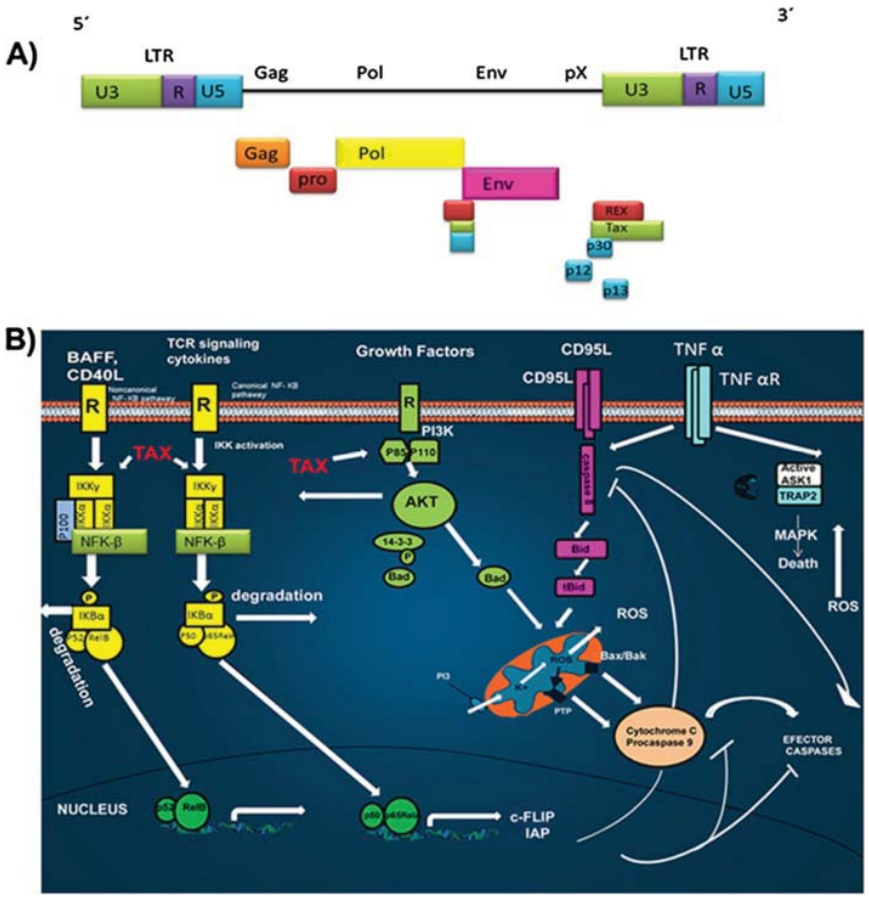

Figure 4 HTLV-1 and apoptotic signaling pathways. A) The HTLV-1 viral genome. The genome consists of a single- positive strand of RNA. The long terminal repeats (LTRs) flank the ORF (boxes) of the structural (orange, red, yellow and pink) and the nonstructural (blue, green) viral proteins. **B**) Main death pathways controlled by HTLV-1 proteins. In infected cells receptor-mediated death (through CD95/Fas) is inhibited by a Tax/NF-kB-mediated upregulation of c-FLIP and IAPs. The PI3K-AKT pathway, also activated by Tax, acts by inhibiting the pro-apoptotic protein Bad and by activating the NF-kB pathway. The accessory proteins, p12 and p13, regulate Bcl-2 and caspases 3 and 9, and also ROS production by mitochondria.

associates with the NS4A cofactor protein, a complex is formed. This complex localizes in the mitochondria and participates in the release of cytochrome c and the activation of caspase 8 [107]. The functions of NS5A are not well defined yet; but it is thought to interfere with the response to IFN and may participate in viral replication. With respect to its role in apoptosis, this protein has sequences homologous to bcl-2 and binds to FKBP38, increasing the anti-apoptotic effect of Bcl-2. Conversely, it has been demonstrated that NS5A inhibits the pro-apoptotic activity of Bax in hepatocytes cells [108]. The anti-apoptotic effect of NS5A is also mediated by the recruitment of p53 in the cytoplasm, the activation of STAT3, and the increase in the expression of Bcl-XL and p21.

The impact of the induction of apoptosis in chronic HCV infection not well understood. Almost for each of the viral protein studied, according to the experimental model, pro-apototic and anti-apoptotic effects have been identified. The modulation of apoptosis by HCV proteins is an important issue to study in order to understand its role in acute HCV infection and persistence.

Human t-cell leukemia virus type 1 (human t-lymphotropic virus type 1)

Currently, there are close to 20 million people infected with the Human T-Cell Leukemia virus type 1 (HTLV-1) worldwide and between 3 to 5% of these individuals develop diseases related to this infection [109].

HTLV-1 is a member of the *Retroviridae* family, which is in the *Oncovirus* subfamily. It is a RNA retrovirus that is involved in carcinogenic processes due to its participation in malignant adult T-cell leukemia. Additionally, it

is involved in the development of a subacute myelopathy, termed HTLV-1 associated myelopathy [110].

Viral genome and structure

HTLV-1 mainly infects CD4+ T-lymphocytes; once the infection has been established, it can remain integrated in the host in the form of a provirus. HTLV-1 has a relatively small genome of 9 kb, comprising the structural and enzymatic genes gag, pro, pol, and env, which are flanked by two terminal regions of repeated sequences (LTRs) (Figure 4A) [108]. The long terminal repeat (LTR) region is subdivided into three regions, U3, R and U5 and contains cis-active elements that are essential for the transcription and expression of viral genes. The pX region contains four open reading frames (ORFs), that encode the accessory proteins ($p12^{I}$, $p13^{II}$, $p30^{II}$), the posttranscriptional regulator REX (ORF III) and the transactivator Tax (ORF IV) [111]. The regulatory proteins Tax and HBZ play a particularly important role in viral persistence and pathogenesis.

Role of Tax in apoptosis

Tax is a nuclear protein encoded by HTLV-1 that has been implicated in viral replication, because it is a transcriptional activator of the LTR. This protein participates in infection, cell proliferation and cell survival [112]. Tax can also activate transcription factors, such as: NF-κB, CREB, SRF, and AP-1.

Tax suppresses a wide wide range of pro-apoptotic factors and induces the expression of apoptosis inhibitors. Tax regulates important signaling pathways, such as the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB), and Akt, both anti-apoptotic proteins which are currently being studied as possible targets for the treatment of adult T-cell leukemia/lymphoma (ATLL) (Figure 4B) [113]. NF-κB is regulated by a family of inhibitors, IkappaB, that retain NF-κB in the cytoplasm, thus preventing its function. The phosphorylation of IkappaB inhibitors by the IKK complex leads to their ubiquitination and degradation, thus activating NF-κB [114]. This effect induces the transcription of a series of anti-apoptotic proteins, such as the Bcl-xL [115] and expression of IAP proteins [116]. Tax activates IKK [117] and can form complexes with the IKKα / IKKγ proteins, thus activating NF-κB [118]. Additionally, Tax can directly regulate the transcription of CBP/p300, a transcriptional coactivator of NF-κB [117-119].

Tax also modulates the signaling pathway regulated by Akt, which is constitutively active in the majority of patients with ATLL [120]. Akt induces the activation of transcription factors, such as AP-1 and β-catenin [121], leading to expression of Bcl-xL, the repression of p53, and overall cell survival.

In addition to the structural proteins, HTVL-1 encodes two accessory proteins, p12 and p13, that have been implicated in the regulation of Bcl-2 family members and caspase 3 and 9 (Figure 4B) [122].

The Epstein-barr virus

The Epstein-Barr virus (EBV) belongs to the gamma-1 subfamily of the herpes virus, also called *lymphocriptovirus* (LCV). The LCVs only affect primates and EBV is the only member that infects humans. EBV was initially isolated from Burkitt lymphoma (BL) cells [123]. After primary infection, this virus can establish long-term latent infections in B-lymphocytes. EBV has been associated with a number of lymphoid and solid tumors in both immunocompetent and immunocompromised individuals.

Viral genome and structure

The Epstein-Barr virus has a linear, double-stranded DNA genome of approximately 184 kb that is wrapped inside a protein capsid (Figure 5). Its DNA contains a short U_S and a long U_L domain that encode the majority of its viral proteins, the internal region, IR1, and the terminal tandem repeat region, TR. When the virus infects a cell, which typically only requires a single virion, the ends of the linear genome bind to each other and persist as episomal DNA [124]. During the latent phase, there is no production of EBV virus and only a small number of viral genes are expressed. These genes affect the normal B cell growth mechanisms, leading to the immortalization of the cells [125]. The latent infection of immortalized B cells is associated with six nuclear antigens, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and the leader protein EBNA-LP; three membrane proteins, LMP-1, -2A and -2B; two small nuclear RNAs, EBER1 and EBER2; and transcripts from the BART region, which encodes the majority of the EBV micro RNAs (miRNAs) [126]. The expression of the complete repertoire of viral latent genes is referred to as Latency III [127,128].

The BZLF1 and BRLF1 proteins are key mediators of the transition from the latent cycle to the lytic cycle transition. These proteins are transactivators for other genes related to the lytic cycle and induce the expression of the viral DNA polymerase. To induce the replication, approximately 80 viral proteins are expressed during the lytic phase, including transcriptional activators, DNA replication factors, and structural proteins, such as the antigens that form the viral capsid.

EBV and apoptosis

The fact that EBV positive BL tumor cells present the virus in a latent form strongly suggests that EBV is essential for the survival of BL cells *in vivo*. Even though the virus can be eliminated from BL cells in culture through continuous passages, the direct elimination of

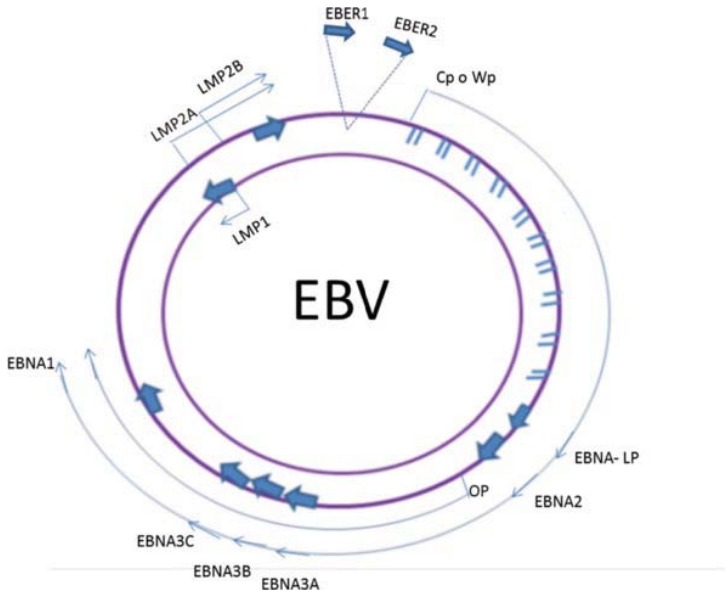

Figure 5 EBV genome. Location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The bottom arrows represent coding exons for each of the latent proteins; the latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A, 2B). EBNA-LP is transcribed from variable numbers of repetitive exons. The arrows with the discontinuous lines close together at the top represent the highly transcribed nonpolyadenylated RNAs EBER1 and EBER2; their transcription is a consistent feature of latent EBV infection. EBNAs are transcribed from either the Cp or Wp promoter.

EBV from these cells induces apoptosis [129]. EBNA1, the EBERs, and the viral miRNAs have all been proposed to be involved in BL cell proliferation and/or resistance to apoptosis, thus conferring a selective advantage to neoplastic cells. There is evidence that EBNA1 has an anti-apoptotic effect in BL cells [124], but the mechanism has yet to be elucidated. Some studies suggest have suggested that the EBERs and EBNA1 are sufficient to promote the malignant growth of BL cells *in vivo*, even in the absence of any other latent phase EBV proteins [129,130].

PKR is a central effector of many apoptotic and stress signaling pathways, and is activated through diverse stimuli, including dsRNA. EBER1 has been shown to be an inhibitor of PKR [131]. The EBERs are dsRNA molecules that have the ability to inhibit PKR activity by binding to it, thus preventing further interactions with other dsRNA molecules and precluding the induction of antiviral and apoptotic pathways. The role of EBER in PKR inhibition during tumorigenesis has not been elucidated. However, the tumorigenic potential of cells that express inactive PKR has been clearly documented [132]. In addition to inhibiting PKR, EBERs have been implicated in apoptosis resistance via the alteration of the expression of the central anti-apoptotic factor, Bcl-2. Initial studies have shown that BL clones expressing EBER also have increased expression of Bcl-2 [133].

Moreover, during the EBV infectious cycle, the viral protein LMP1 has been proposed to mimic the signaling induced by CD40 by providing erroneous survival signals in infected B cells within the germinal center [134]. LMP1 can contribute to neoplastic transformation and to tumor progression by modulating the TNF receptor pathway, through its interaction with the CTAR1 and CTAR2 domains in a ligand-independent manner [135]. In turn, these domains interact with the factors associated with TNF-R (TRAFs) and the death domains coupled with TNF-R (TRADDs) [136]. The association of LMP1 with the TRAF and TRADD molecules activates a signaling cascade that results in the constitutive activation of the JNK, NFKB and PI3K pathways. The activation of these key-signaling pathways increases cellular growth and promotes survival through the induction of anti-apoptotic factors, including Bcl-2 and A20.

Kaposi's sarcoma Herpesvirus

Kaposi's sarcoma (KS) is a malignant, multifocal systemic disease that originates from the vascular endothelium. The disease has a variable clinical course and most frequently manifests as skin lesions. Different clinical forms can be

distinguished, including the so-called classic Kaposi's sarcoma, which results from immunosuppression and often occurs in organ transplant recipients or after long-term cortisone treatment; the endemic African Kaposi's sarcoma; and the epidemic HIV-associated Kaposi's sarcoma. KS is among the most common malignancies occurring in the HIV-infected patients. Although the incidence of AIDS-associated KS has declined since the implementation of highly active antiretroviral treatment (HAART), up to 50% of patients with AIDS-KS never achieve total remission [137]. All types of Kaposi's sarcoma are due to the infection with Kaposi's sarcoma-associated herpesvirus (KSHV), also known as Human Herpesvirus 8 (HHV-8) [138]. While its routes of transmission are not completely understood, important known routes are sexual transmission, saliva, blood or organ transplantation [139]. In addition to KS, KSHV has been associated with lymphoproliferative disorders, including multicentric Castleman's disease (MCD), plasmablastic lymphoma, and primary effusion lymphoma (PEL) [140].

KSHV infects endothelial cells or circulating endothelial and/or hematopoietic progenitors [141]. Its oncogenicity is supported by the numerous pro-angiogenic molecules that are induced following the infection of endothelial cells, including the VEGF-VEGFR family, cyclooxygenase 2 (COX2) and angiogenin [142]. However, in the general population, KSHV infection rarely leads to KS, indicating the need of cofactors, such as immunosuppression, in order for a tumor to be induced.

The KSHV genome

The KSHV genome is a linear, double-stranded DNA of approximately 165 to 170 kb in length [143]. During latency, it may also exist in a circular, episomal form in the host nucleus [144]. Among the viruses that infect humans, KSHV is most closely related to the gammaherpesvirus, Epstein Barr (EBV).

KSHV encodes 87 open reading frames (ORFs) and at least 17 microRNAs, 14 of which co-express as a cluster. KSHV has at least 14 ORFs that encode cellular orthologues that play important roles in controlling the cell cycle and cell signaling [145].

The life cycle of all herpesviruses includes prolonged latent and lytic phases. Reactivation occurs when the promoter of ORF50 is activated and the replication and transcription activator RTA is expressed, which is the main regulator of the lytic replication program [145]. During the latent phase, a subset of genes are expressed, such as the latency-associated nuclear antigen (LANA), vCyclin, vFLIP, kaposins and KSHV-encoded 17 miRNAs, which are derived from the processing of 12 pre-miRNAs [146]. These genes are required for viral episome maintenance, host cell survival, and the suppression of lytic gene activation [147]. These protein increase proliferative signals, decrease apoptosis and induce the activation proangiogenic and inflammatory signals, as well as limitless replicative potential.

The role of KSHV in apoptosis

Latent phase proteins

The multifunctional protein, LANA, maintains the viral episome and can also interfere with important cellular processes. The main functions of KSHV latent proteins are exposed in Table 3. LANA is considered to be an oncogenic protein due to its ability to dysregulate tumor suppressor pathways associated with p53 and pRb and to transform primary rat embryo fibroblasts in cooperation with the cellular oncogene H-ras [148]. In addition, this protein has been shown to deregulate Wnt signaling by altering the subcellular distribution of glycogen synthase kinase 3 (GSK-3), a negative regulator of β-catenin [149]. LANA modulates apoptosis by direct binding to p53 (Figure 6). It also associates with different host cell proteins, including chromatin-associated proteins, which are involved in the epigenetic silencing of TGFβ expression. These associations have antiproliferative and apoptotic effects on epithelial, endothelial, and hematopoietic cell lineages [150] Table 3.

vCyclin (viral homolog of cellular cyclin D) is a constitutive activator of cyclin dependent kinase 6 (CDK6). The expression of vCyclin and the formation of the complex, vCyclin/CDK6, leads to defects in cytokinesis, which result in polyploidy and the activation of p53 [152]. However, in the absence of functional p53, cells survive, exposing the oncogenic role of vCyclin. Substrates of the vCyclin/CDK6 complex include pRb and p27 [153]. As such, vCyclin efficiently accelerates cell-cycle progression, even in the presence of CDK inhibitors. In contrast, it has been demonstrated that the expression of vCyclin in cells with increased levels of CDK6 triggers apoptosis independently of p53 and pRb. These findings suggest that vCyclin may have both growth-promoting and apoptotic functions in the development of Kaposi's sarcoma.

vFLIP (viral FLICE inhibitory proteins) is a small polypeptide composed of two tandem death effector domains (DEDs). The protein is homologous to the cellular FLIP proteins, which are also called FLICE, and blocks the signaling of caspase-8 (Figure 6). This protein could be recruited to DISC through the interaction of its tandem DEDs with DED. As such, FLIP excludes procaspase 8 from the DISC complex [154].

Several KSHV miRNAs have also been shown to modulate host gene expression, suggesting some roles for the miRNAs in the pathogenesis of malignancies induced by KSHV [165]. The target of miR-K5 is the Bcl2 associated factor, BCLAF1, which promotes

Table 3 Functions of KSHV viral proteins

Latent phase proteins	Functions
LANA 1,2	Establish and maintain the latency in KSHV infected cells, bind directly to p53 and pRb [148].
Kaposin A, B	Induce the expression of growth factor receptors, possible transformation activity [151].
vcyclin	Forms a complex with CDK-6 to inactivate the Rb protein, promoting cell cycle progression and proliferation [152]. Induces apoptosis independent of p53 [153].
vFLIP	Blocks caspase 8 activation [154], potent activator of NFκB [154].
Lytic phase proteins	
ORF50 (RTA)	Regulates the lytic replication [155].
K1	Activator of the molecules that mimic signaling via the B cell antigen receptor [156].
K8	Regulates lytic-cycle DNA replication [157].
K3, K5	Mediate the down regulation of several immunomodulatory proteins, including CD86, intercellular adhesion molecule 1 (ICAM-1; CD54), and IFN-R [158].
vIL-6	Induces angiogenesis and tumorigenesis by regulating PI3K/PTEN/AKT/GSK-3β signaling pathway [159].
vIRF-1	Binds and inhibits pro-death activities of proteins Bid and Bim [160].
vMIPs	Binds to chemokine receptors and induce angiogenesis [161].
vGPCR	Transformation activity; promotes the secretion the growth factors, such as VEGF, bFGF, IL-8, and IL-6 [162].
vBcl-2	Inhibits apoptosis [163].

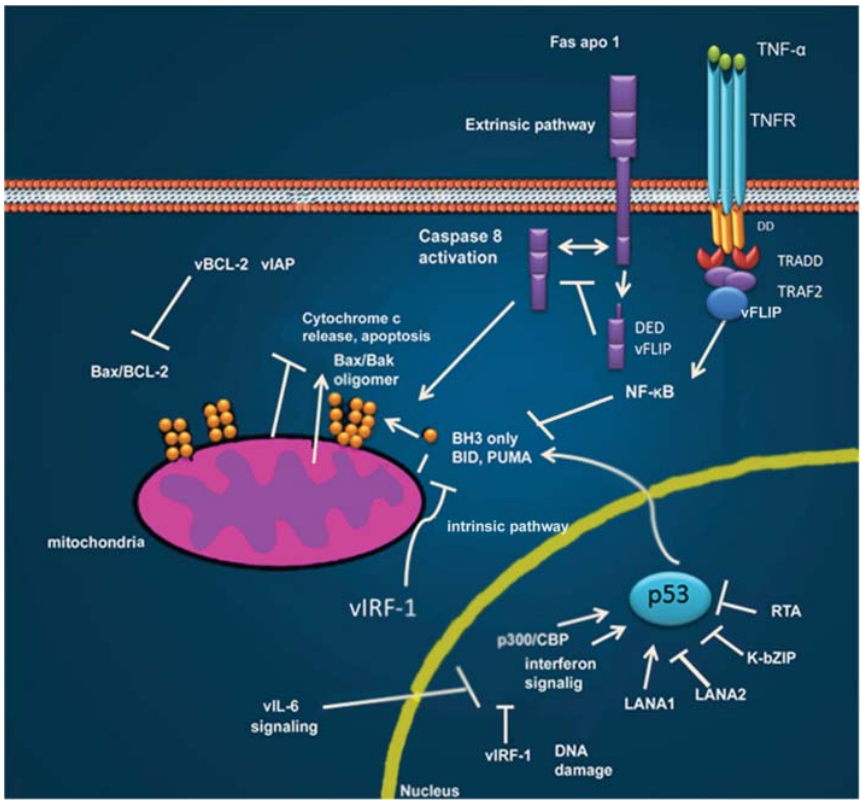

Figure 6 Different KSHV proteins inhibit intrinsic and extrinsic apoptotic pathways. vFLIP directly binds to death effector domains, or to the TRAF complex, inhibiting activated-Fas signaling, or activating NF-kB. Both vIAP and vBCL-2 act at the mitochondria to stabilize the mitochondrial membrane and inhibit the activating effects of BH3-only pro-apoptotic molecules.vCyclin LANA1, LANA2, K-bZIP and RTA inhibit p53-induced apoptosis either through direct binding or through inhibition of the p300/CBP coactivator used in p53 transcriptional signaling. vIRFbinds and inhibits pro-death activities of proteins Bid and Bim. Modified from Moore P.S. et al. (2007) [164].

apoptosis [166]. MiR-K1 targets IκBα, an inhibitor of NF-κB, which inhibits the activation of lytic viral promoters [167].

Lytic phase proteins

The aberrant expression of the ORF50 protein is required for the initiation of the lytic phase and the expression of many KSHV-encoded lytic genes, such as K1, K3, and K5; viral macrophage inflammatory proteins (vMIPs); K12; viral G-protein-coupled receptor (vGPCR); viral dihydrofolate reductase (vDHFR); DNA replication factors; and thymidylate synthase [168].

Other lytic proteins that are important in cellular transformation are the viral orthologues of cellular proteins such as viral interleukin-6 (vIL-6), vBCL-2, vIRF and vCCLs, whose functions are summarized in Table 3. vBCL-2 inhibits apoptosis through the inhibition of pro-apoptotic BH3 domain-containing proteins (Figure 6) [169,170]; while vIRF1 inhibits p53-induced apoptosis through its interaction with the central DNA-binding domain of p53 and with the upstream ATM kinase [170].

K1, which is the first ORF of KSHV, inhibits apoptosis by inducing the release of growth factors such as VEFG, leading to the subsequent activation of the PI-3 K-AKT pathway. Prior to cell lysis, the inhibition of apoptosis by lytic proteins could also contribute to cell transformation, viral replication and virion production and assembly [171].

Conclusions

With the acceptance that tumor viruses account for a substantial fraction of human cancers, tumor virology has evolved from a niche area of research to a central and active field of cancer research. The recent development of powerful new virus detection methods may further extend the spectrum of virus-associated cancers in the future. Cancers exhibiting epidemiological features that are compatible with an infectious cause and cancers that are linked to immunosuppression, are particularly interesting candidates to screen, with the goal of identifying new tumor viruses. Tumor viruses represent promising targets for specific preventive and therapeutic anticancer strategies, as evidenced by the success of the HBV and HPV vaccines. These findings should further motivate research on improved or novel prophylactic vaccines that may protect against other tumor viruses. The deeper understanding of the biology of oncogenic viruses and the defense mechanisms of the host should also facilitate the development of specific therapeutic approaches, because viruses represent targets that are unique to diseased cells.

Successful viral replication requires not only the efficient production and spread of viral progeny, but also the evasion of host defense mechanisms that limit viral replication by killing the infected cells. In addition to inducing immune and inflammatory responses, most viruses encode proteins that interact with the biochemical pathways regulating apoptosis of the infected cell. For some viruses, the inhibition of apoptosis seems to be essential for the maintenance of viral latency. For other viruses, the carefully choreographed induction of apoptosis during infection may represent the basis for cytotoxicity and be an important outlet for the dissemination of virus progeny. For non-lytic virus, pro-apoptotic effects could be implicated in a properly completion of the viral cycle. As these processes are understood in greater detail, the opportunities for the development of new drugs to combat clinically important viruses will almost certainly arise. Such drugs could promote the early death of infected cells, inhibit virus release or, in the case of latent viruses, manipulate the latency switch to minimize the effects of infection.

As the infection mechanisms of oncogenic viruses are better characterized, remarkable insights into the molecular biology of apoptosis will be forthcoming.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AMFG prepared the Human Papillomavirus chapter, ACP prepared the Hepatitis Viruses chapter and participated in the Kaposi's Sarcoma Herpesvirus chapter, JMM prepared the HTLV-1 chapter, ML participated in the design and coordination of the manuscript and prepared the Epstein-Barr Virus and the Kaposi's Sarcoma Herpesvirus chapters. All authors read and approved the final manuscript.

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