ΔNp63α A KEY FACTOR OF EPITHELIAL DIFFERENTIATION CONTROLS THE ACTIVITY OF YB-1 ONCOPROTEIN: POTENTIAL IMPLICATIONS IN CARCINOGENESIS

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Premesse scientifiche e metodologiche

p63 (TP63/TP73L) è un omologo dell'oncosoppressore p53 e rappresenta il capostipite della famiglia genica di p53 (Yang A. et al., 1998; Osada M. et al., 1998). Il locus genico di p63, grazie all'utilizzo di due distinti promotori e ad eventi di splicing alternativo che si verificano a carico dell'estremità C-terminale, dà origine ad un totale di sei isoforme proteiche. Tre di queste possiedono all'estremità N-terminale un dominio di attivazione trascrizionale (TA) e sono funzionalmente simili a p53 (TAp63α, β e γ), le altre tre isoforme mancano del dominio TA e sono in grado di promuovere la sopravvivenza e la proliferazione cellulare (ΔNp63α, β e γ) (Schmale et al., 1997). L'isoforma TAp63γ è la più simile a p53, mentre le isoforme α possiedono una notevole estensione al C-terminale, che include un dominio conservato di interazione proteina-proteina noto come "Sterile Alpha Motif" (SAM). L'analisi dei topi knockout dimostra inequivocabilmente che p63 esercita un ruolo cruciale nello sviluppo dei tessuti epiteliali fra i quali l'epidermide e di organi derivati da interazioni epitelio-mesenchima quali le ghiandole salivari, lacrimali, mammarie e la prostata (Yang A. et al., 1999). ΔNp63α guida attivamente le fasi precoci dello sviluppo epidermico e governa il destino dei cheratinociti dello strato basale (Romano et al., 2009). I cheratinociti proliferanti dello strato basale, nell'epidermide dell'adulto, esprimono alti livelli di ΔNp63α e l'iper-espressione di detta proteina è strettamente associata all'insorgenza nell'uomo di neoplasie a carico dell'epidermide stessa (Parsa et al., 1999). Tuttavia, il ruolo che ΔNp63α riveste nello sviluppo e nella progressione del tumore, con particolare riguardo al processo di metastatizzazione, è piuttosto controverso. Per esempio, l'assenza dell'espressione di ΔNp63α è caratteristica di tumori anaplastici della vescica, dell'uretere e del seno (Urist M.J. et al., 2002; Koga F. et al., 2003; Wang X. et al., 2002) e l'over-espressione di mutanti di splicing di ΔNp63α è stata osservata in numerosi carcinomi squamosi metastatici (Westfall and Pietenpol 2004). Inoltre, in cellule di carcinoma squamoso, il fattore di trascrizione Snail1 che promuove transizione epitelio-mesenchima (EMT), reprime ΔNp63α favorendo dunque la metastatizzazione del tumore (Herf et al. 2010).
L'attività funzionale di ogni proteina è legata alle sue interazioni con altre molecole e, nelle cellule eucariotiche, alla sua residenza nel corretto compartimento subcellulare poiché solo nel compartimento corretto troverà i "partner" corretti. Nel laboratorio in cui ho svolto la mia tesi è stata intrapresa una linea di ricerca volta ad identificare gli interattori di p63 con

l'obiettivo di chiarire i meccanismi molecolari che coinvolgono ΔNp63α nello sviluppo e nella progressione del tumore. La comprensione dei meccanismi tramite i quali p63 controlla la genesi e progressione del tumore così come la patogenesi delle displasie ectodermiche costituirà una tappa fondamentale per lo sviluppo di nuovi farmaci e strategie terapeutiche. Mediante un approccio di proteomica funzionale, sono stati identificati 53 potenziali interattori di ΔNp63α. Fra questi è inclusa l'oncoproteina YB-1. YB-1 è un importante marcatore tumorale per il cancro al seno, il carcinoma e l'adenocarcinoma polmonare, l'osteosarcoma, il carcinoma del colon-retto ed il melanoma (Wu et al. 2007).

L'obiettivo del mio lavoro è stato quello di comprendere il ruolo funzionale dell'associazione fra ΔNp63α e YB-1. E' già noto infatti, che YB-1 interagisca funzionalmente con p53 e con esso trasloca nel nucleo regolando l'espressione genica p53-dipendente. YB-1 controlla la trascrizione e la traduzione di molti fattori effettuando "shuttling" tra nucleo e citoplasma. Nel nucleo YB-1 regola la trascrizione di geni coinvolti nella chemio-resistenza, direttamente o attraverso l'associazione con p53, AP-1, Smad3 o p300 (Bader 2005), e di geni pro-proliferativi come cyclin A, cyclin B1, DNA pol α and PI3KCA, la subunità catalitica della fosfatidilinositolo-3-chinasi (PI3K) (Jurchott 2003; En-Nia 2005; Astanehe 2009). Nel citoplasma YB-1 svolge una funzione più strutturale ed è coinvolta nell'organizzazione spaziale dei complessi mRNPs (Skabkin, M. A 2004). In particolare, legandosi al Cap 5' dei trascritti scalza i fattori di inizio della traduzione eIF4E ed eIF4G causando blocco della traduzione dell'mRNA. D'altra parte, YB-1 può legare ed attivare la traduzione cap-indipendente di specifici messageri coinvolti nell'EMT (transizione epitelio-mesenchima) come quello codificante SNAIL1, fattore di trascrizione che blocca l'espressione delle E-caderine (Battle et al., 2000). Nel cancro al seno, appunto, i livelli di espressione della proteina YB-1 sono correlati ad una ridotta espressione di E-caderina e ad una prognosi infausta (Evdokimova 2009). Inoltre, la iper-espressione di YB-1 in linee cellulari mammarie non invasive induce transizione epitelio-mesenchima (EMT) accompagnata da aumento del potenziale metastatico e ad un ridotto livello di proliferazione (Mouneimme G., 2009).

La mia ricerca si è dunque articolata nelle seguenti quattro fasi:

I. Studio dell'interazione tra le isoforme di p63 e YB-1 mediante approcci *in vitro* ed in diversi contesti cellulari (Saos2, U2OS, H1299, HaCat, Hela, MDA231 e MCF7).
II. Determinazione dell'effetto reciproco sui livelli di espressione, sulla stabilità e sulla localizzazione sub-cellulare attraverso iper-espressione e silencing specifico, immuno-localizzazione

e microscopia a fluorescenza, e misure di "protein-decay" in cellule trattate con inibitori della sintesi proteica.

III. Analisi dell'effetto dell'interazione con p63 sulle funzioni nucleari e citoplasmatiche di YB-1 mediante tecniche di immunoprecipitazione della cromatina e dell'RNA, tecniche di Real Time PCR e saggi luciferasi.

IV. Determinazione dell'effetto di p63 e YB-1 sulla proliferazione e migrazione cellulare. Analisi di espressione in cheratinociti primari e di epitelio di cornea in fase rigenerativa e non.

Risultati

I. Mediante saggi di coimmunoprecipitazione, effettuati in diverse linee cellulari quali le Saos2, U2OS, H1299, HaCat, Hela, MDA231 e MCF7, ho potuto dimostrare che le proteine p63 e YB-1 interagiscono. Tale interazione è stata verificata sia per proteine espresse in maniera endogena sia per proteine esogene espresse in maniera transiente mediante trasfezione. Ho potuto inoltre mettere in evidenza che la proteina YB-1 interagisce specificamente con le isoforme α, ma non γ, di p63 svelando quindi l'importanza della regione carbossi-terminale α-specifica di p63 nell'interazione con YB-1. Al fine di stabilire se l'interazione dimostrata fosse diretta, i cDNA codificanti le proteine ΔNp63α ΔNp63γ e YB-1 sono stati sub-clonati in vettori per l'espressione in *E.coli.* Le proteine sono state espresse e purificate per cromatografia di affinità ed utilizzate in esperimenti di Far-western. Tale saggio ha confermato, *in vitro,* che YB-1 interagisce specificamente con l' isoforma ΔNp63α e non con la γ e che tale interazione è appunto diretta.

II. E' stata, quindi, analizzata la capacità delle diverse isoforme di p63 di influenzare i livelli di espressione di YB-1, e viceversa. Come osservato, p63 e YB-1 non esercitano alcun controllo reciproco sulla loro espressione genica. Ciò è stato dimostrato sia a livello di mRNA mediante l'approccio della Real Time PCR sia a livello di proteina mediante esperimenti di silencing o di iper-espressione transiente. Tuttavia, l'analisi della emivita della proteina YB-1 ho potuto mettere in evidenza che essa è di gran lunga superiore in cellule che esprimono ΔNp63α rispetto a cellule che ne sono prive. Ciò è vero sia nel caso in cui la proteina ΔNp63α sia endogenamente espressa, sia nei casi in cui la proteina venga espressa in seguito a trasfezione. YB-1 è un fattore pleiotropico coinvolto in numerosi processi cellulari che vanno dal controllo della trascrizione al controllo della traduzione di specifici messaggeri. Sebbene in condizioni normali YB-1 sia localizzata prevalentemente nel citoplasma, essa è in grado di traslocare dal citoplasma al nucleo e viceversa. Dal momento che p63 è un fattore trascrizionale

localizzato prevalentemente in sede nucleare, mi sono chiesto quale fosse la localizzazione di YB1 in presenza di ΔNp63α. Per rispondere a tale quesito ho analizzato la localizzazione sub-cellulare di YB1, in assenza ed in presenza di p63, mediante fluorescenza diretta e l'uso della proteina YB-1 fusa all'epitopo GFP. Parallelamente ho svolto saggi di localizzazione cellulare mediante immunofluorescenza sulla proteina YB-1 endogenamente espressa. Tali esperimenti sono stati condotti in cellule derivate da cancro del seno dove l'iperespressione di YB-1 è nota indurre un fenotipo tumorale aggressivo. Ho potuto osservare che ΔNp63α è in grado di determinare accumulo di YB-1 in sede nucleare. Il dato è stato confermato anche mediante la messa a punto di un protocollo per la separazione differenziale in gradiente di saccarosio degli estratti citoplasmatici e nucleari. Mediante questo approccio ho potuto notare che l'espressione dell'isoforma ΔNp63α causa attivazione della chinasi pAkt. Questo aspetto è particolarmente interessante in quanto il pathway di PI3K/Akt(PKB) è noto giocare un ruolo chiave nella genesi e progressione di molte forme tumorali. YB-1 è un noto target della chinasi Akt che lo fosforila sulla serina in posizione 102. Tale evento di fosforilazione determinerebbe una modifica conformazionale di YB-1, cui fa seguito il suo distacco dagli mRNA citoplasmatici e la sua traslocazione nucleare. Dal momento che ΔNp63α induce attivazione di Akt era ragionevole presupporre che l'accumulo nucleare di YB-1, mediato da p63, potesse dipendere dall'attivazione della chinasi Akt. Questo aspetto è stato affrontato mediante due diversi approcci:

i) Iper-espressione del mutante costitutivamente attivo di Akt.

ii) Utilizzo del mutante fosfo-mimetico (S102D) e del mutante fosfo-difettivo (S102A) di YB1. I risultati indicano che l'attivazione di Akt non è sufficiente a promuovere la traslocazione nucleare di YB-1 e che la presenza fisica di ΔNp63α è necessaria e sufficiente a promuovere anche l'accumulo del mutante di YB1 non fosforilabile da Akt.

III. Come precedentemente accennato, YB-1 opera nel nucleo come fattore trascrizionale. Un noto *target* di YB-1 è il gene PI3KCA, codificante la sub-unità catalitica dello fosfotidil-inositolo-3-chinasi. Attraverso la tecnica della immuno-precipitazione della Cromatina (ChIP) abbiamo dimostrato che in presenza di ΔNp63α aumenta il legame di YB-1 al promotore del gene PI3KCA. Inaspettatamente, abbiamo osservato che la stessa proteina ΔNp63α è in grado di legare il promotore PI3KCA. Mediante saggi reporter (luciferasi) abbiamo dimostrato che ΔNp63α è un attivatore trascrizionale di PI3KCA e che il silenziamento di YB-1 riduce l'attività di p63 su questo promotore. Questo dato indica che YB-1 e p63 cooperano nella

regolazione dell'espressione della proteina PI3KCA. Per quanto concerne le attività citoplasmatiche, è noto che YB1 promuova la traduzione cap-indipendente dei trascritti di Snail1 e Twist, due fattori coinvolti nella transizione epitelio-mesenchima (EMT). Mediante saggi di immunoprecipitazione dell' RNA con anticorpi diretti contro YB-1 ho potuto osservare una riduzione della quantità di YB-1 legata al trascritto di Snail1, in cellule esprimenti p63. Un risultato analogo è stato ottenuto sul trascritto di YB-1, sul quale si lega la stessa proteina YB-1. Coerente con quanto osservato negli esperimenti di RNA-IP, i livelli di proteina Snail 1 appaiono ridotti e si verifica un aumento della E-caderina in presenza di ΔNp63α, la riduzione di Snail non riguarda il trascritto la cui abbondanza, misurata mediante esperimenti di rt-PCR, risulta invariata. Per contro ho potuto costatare una diminuzione del trascritto di Twist in presenza di ΔNp63α. Al fine, dunque, di dimostrare se quanto osservato a livello molecolare avesse un effetto in termini di mobilità cellulare abbiamo condotto saggi di migrazione con microscopia "*single cell tracking*" utilizzando cellule H1299 ΔNp63α Tet-on, in cui cioè l'espressione di ΔNp63α è inducibile mediante aggiunta di doxiciclina nel mezzo di coltura. In collaborazione con il gruppo del Prof. Netti abbiamo eseguito misure di velocità di migrazione e persistenza cellulare. Quest'ultimo parametro esprime il tempo in cui una cellula migra in una stessa direzione. Le cellule H1299/ΔNp63α TET-on sono state trasfettate con il vettore codificante la proteina YB1-GFP. Un numero statisticamente significativo di cellule fluorescenti è stato seguito in "Time lapse" per misurarne la velocità di migrazione e la persistenza. Le cellule iper-esprimenti YB-1 presentavano un incremento della velocità media di migrazione pari ad un fattore di 1.3 ed una riduzione di persistenza pari al 40% del controllo (cellule trasfettate con il vettore esprimente la sola GFP). Per contro, le cellule esprimenti YB1-GFP e ΔNp63α (+dox) si comportano in maniera quasi sovrapponibile al controllo, indicando che p63 è in grado di revertire il fenotipo indotto dalla iper-espressione di YB1. Infine, grazie ad una collaborazione con il Dott. Enzo Di Iorio della Banca degli occhi Veneta, abbiamo esaminato l'espressione di YB-1 e ΔNp63α nell'epitelio corneale a riposo ed in fase rigenerativa (dopo una lesione) mediante tecniche di immunoistochimica. Abbiamo osservato la localizzazione prevalentemente nucleare per p63 e quella citoplasmatica per YB-1. Peraltro, è frequente la sovrapposizione dei segnali di YB-1 e p63 particolarmente in quelle cellule che si spostano dallo strato basale dell'epitelio corneale a quello sovrastante dove perdendo le caratteristiche staminali si avviano al differenziamento terminale. Ulteriori studi saranno necessari

per comprendere a fondo il ruolo dell'associazione di p63 e YB1 nella rigenerazione di tale tessuto. In conclusione, durante il mio lavoro di dottorato, ho dimostrato l'esistenza di un complesso ΔNp63α/YB-1 in grado di esercitare un'attività di controllo sia sul pathway di PI3K/Akt sia su geni che attivano la transizione epitelio-mesenchima. Ambedue questi pathway sono coinvolti nel processo di tumorigenesi e progressione tumorale. Il mio lavoro getta le premesse per uno studio volto ad identificare nuove molecole e strategie terapeutiche per contrastare il processo tumorale.

Abstract

Scientific and Methodological premises

The p63 (TP63/TP73L) gene, a homologue of the tumor-suppressor p53, is the founder member of the p53 gene family (Yang A. et al., 1998; M. Osada et al., 1998). Using two different promoters and alternative splicing events at the C-terminal end, the p63 locus gives rise to a total of six protein isoforms. Among them, three exhibit an N-terminal transcriptional activation domain (TA) and are functionally similar to p53 (TAp63 α, β and γ) while the remaining lack the TA domain and are able to promote survival and cell proliferation (Δ Np63 α , β and γ) (Schmale et al., 1997). TAp63γ is most similar to p53, while the α isoforms possess a considerable extension to the C-terminus including a conserved protein-protein interaction domain known as "Sterile Alpha Motif" (SAM). The analysis of knockout mice demonstrates unequivocally that p63 exerts a crucial role in the development of epithelial tissues including the skin and organs derived from epithelial-mesenchymal interactions such as salivary glands, lacrimal, mammary and prostate (Yang A. et al., 1999). ΔNp63α actively guides the early stages of development and governs the fate of the epidermal basal layer (Romano et al., 2009). In adult epidermis, the basal layer contains proliferating keratinocytes that express high levels of ΔNp63α. Deregulated expression of ΔNp63α was found to be closely associated with skin cancer in humans (Parsa et al., 1999). However, the role that ΔNp63α plays in cancer development and progression, with particular regard to the metastasis process, is quite controversial. For example, the absence of the expression ΔNp63α is characteristic of anaplastic tumors of the bladder, ureter and breast cancer (Urist MJ et al. 2002; F. Koga et al. 2003; X. Wang et al., 2002) and the over- expression of mutant splicing ΔNp63α has been observed in many metastatic squamous cell carcinomas (Westfall and Pietenpol 2004). Furthermore, in squamous cell carcinoma, the Snail1 transcription factor that promotes epithelial-mesenchymal transition (EMT), represses ΔNp63α thus favoring tumor metastasis (Herf et al. 2010).
The functional activity of each protein is linked to its interactions with other molecules and, in eukaryotic cells, at his location in the correct sub-cellular compartment. In fact, only in the right compartment a protein will make interaction with the correct "partners".
In the lab where I did my thesis, a functional proteomic approach was undertaken to clarify the role played ΔNp63α in

cancer pathogenesis and progression. Understanding the mechanisms by which p63 controls the genesis and progression of tumors as well as the pathogenesis of ectodermal dysplasias will represent a milestone for development of new drugs and therapeutic strategies.
The YB-1 oncoprotein was identified among the potential ΔNp63α interactors. YB-1 is an important tumor marker for breast cancer, lungcarcinoma, adenocarcinoma, osteosarcoma, colorectal cancer and melanoma (Wu et al. 2007).
The goal of my work was to understand the functional role of the association between ΔNp63α and YB-1. YB-1 was already known to functionally interact and translocate with p53 to the nucleus to regulate p53-dependent gene expression. YB-1 controls the transcription and translation of many factors "shuttling" between the nucleus and cytoplasm. Into the nucleus, YB-1 regulates the transcription of genes involved in chemo-resistance, directly or through the association with p53, AP-1, Smad3 or p300 (Bader 2005), and pro-proliferative genes such as cyclin A, cyclin B1, DNA polα and PI3KCA, the phosphatidylinositol-3-kinase catalytic subunit (PI3K) (Jurchott 2003, En-Nia, 2005; Astaneh 2009). In the cytoplasm, YB-1 plays a more structural role being involved in the spatial organization of mRNPs (Skabkin, M. A 2004). In particular, binding to Cap 5 'transcripts compete with eIF4E and eIF4G translation initiation factors blocking translation of specific mRNA. On the other hand, YB-1 can bind and activate cap-independent translation of specific messenger RNAs, such as that of SNAIL1, a factor involved in EMT (epithelial-mesenchymal transition) that blocks the expression of E-cadherin (Battle et at., 2000).
In breast cancer, YB-1 protein expression levels are correlated with reduced E-cadherin expression and a poor prognosis (Evdokimova, 2009). In addition, the YB-1 over-expression in non-invasive breast cancer cell lines induces epithelial-mesenchymal transition (EMT) accompanied by increased of metastatic potential and a reduced proliferation level (Mouneimme G., 2009).
My research is thus divided into the following four phases:
I. Study of the interaction between the p63 isoforms and YB-1 using *in vitro* approaches, in different cell contexts (Saos2, U2OS, H1299, HaCaT, Hela, MCF7, and MDA231).
II. Determination of the mutual effect on protein expression levels and stability. Analysis of sub-cellular localization through immuno-localization and fluorescence microscopy. Measures of "protein-decay" in cells treated with inhibitors of protein synthesis.

III. Analysis of the effect of the interaction with p63 on some YB-1 nuclear and cytoplasmic functions by chromatin and RNA immunoprecipitation, real-time PCR techniques and luciferase assays.
IV. Determination of the effect of p63 and YB-1 interaction on proliferation and cell migration. Analysis of expression in primary keratinocytes and intact or regenerating corneal epithelium.

Results

Through co-immunoprecipitation assays, performed in different cell lines such as Saos2, U2OS, H1299, HaCaT, Hela, MCF7, and MDA231, I demonstrated that p63 and YB-1 proteins interact. This interaction was verified on both endogenous and exogenously expressed proteins. Moreover, I have shown that the YB-1 protein specifically interacts with α but not γ p63 isoforms thus revealing the importance of the p63 α-specific carboxy-terminal in the YB-1 interaction.
In order to establish that the interaction was direct, I subcloned cDNAs encoding ΔNp63α, ΔNp63γ and YB-1 proteins into *E. coli* expression vectors. The proteins were expressed and purified by affinity chromatography and used in Far-western experiments. This assay confirmed, *in vitro*, that YB-1 interacts specifically with ΔNp63α but not γ isoform and that this interaction is direct.
I analyzed the ability of different p63 isoforms to influence the YB-1expression levels, and vice versa. It has to be noted that p63 and YB-1 have no control over their mutual gene expression. This has been demonstrated both at mRNA and protein level by silencing experiments or transient over-expression.
However, I have found that YB-1 protein half-life is much higher in ΔNp63α expressing cells compared to cells lacking p63 expression. This is true in cells expressing endogenous ΔNp63α as well as in those overexpressing exogenous p63.
YB-1 is a pleiotropic factor involved in many cellular processes including the control of transcription and translation. Although under normal conditions YB-1 is mainly localized in the cytoplasm, it is able to move from the cytoplasm to the nucleus and vice versa. Since p63 is a transcription factor localized predominantly in the nuclear, I wondered what was the localization of YB1 in presence of ΔNp63α. To answer this question I analyzed the YB1 sub-cellular localization, in the absence and presence of p63, by direct fluorescence and the use of YB-1/GFP protein. In parallel I've done cellular localization by immunofluorescence assays on the

endogenously expressed YB-1 protein. These experiments were conducted in cells derived from breast cancer, where YB-1 overexpression is known to induce a tumor aggressive phenotype. I have shown that ΔNp63α is able to determine YB-1 accumulation in the nuclear compartment. This was also confirmed by the development of a protocol for differential separation in sucrose gradient of cytoplasmic and nuclear extracts. With this approach I have observed that ΔNp63α expression causes Akt kinase activation. This is an intriguing point since the PI3K/Akt pathway (PKB) is known to play a key role in the genesis and progression of many cancers.

YB-1 is a well known target of Akt kinase. Phosphorylation of Ser102 of YB-1 is supposed to cause a conformational change of YB-1 followed by its detachment from cytoplasmic mRNA and nuclear translocation. Since ΔNp63α induced Akt activation I assumed that YB-1 nuclear accumulation was dependent on Akt kinase activation. I addressed this aspect with two different approaches:

i) Analysis of the effect of constitutively active Akt mutant on YB1 subcellular localization.

ii) Analysis of the effect of ΔNp63α expression on the subcellular localization of the YB1 phospho-mimetic (S102D) or phospho-defective (S102A) mutants.

The obtained results indicate that Akt activation is not sufficient to promote YB-1 nuclear translocation and that ΔNp63α is necessary and sufficient to promote the nuclear accumulation of YB1.

As previously mentioned, YB-1 operates in the nucleus as a transcription factor. A well-known target of YB-1 is the PI3KCA gene, encoding the catalytic subunit of fosfotidil-inositol-3-kinase. Through Chromatin Immuno-Precipitation (ChIP) I have demonstrated that ΔNp63α increases the binding of YB-1 to PI3KCA gene promoter. Surprisingly, I have observed that the same ΔNp63α protein is able to bind the PI3KCA promoter. Using reporter assays (luciferase) we have shown that ΔNp63α is a PI3KCA transcriptional activator and YB-1 silencing reduces the p63 activity on this promoter. This data indicates that YB-1 and p63 cooperate in the control of PI3KCA gene expression.

Concerning the cytoplasmic activities, it is known that YB1 promotes cap-independent translation of Snail1 and Twist transcripts, two factors involved in epithelial-mesenchymal transition (EMT). By RNA immunoprecipitation assays with YB-1 antibodies I have seen a reduction in the amount of YB1-bound Snail1 transcript, in p63 expressing cells. A similar result was obtained on the YB-1 transcript, which is bound by YB-1 protein. Consistent with this, by western blot experiments, I

observed that, in presence of ΔNp63α, Snail 1 protein was reduced while E-cadherin was increase.

By inducing pro-metastatic genes YB-1 increases cell motility. To analyse the effect of ΔNp63α on cell motility I have carried cell migration assays with "single-cell tracking time-lapse microscopy" using H1299 ΔNp63α Tet-on cells, in which the expression of ΔNp63α is inducible by doxycycline.

In collaboration with the Prof. Netti group we performed measurements of cell migration speed and persistence. H1299 ΔNp63α TET-on cells were transfected with the YB1/GFP encoding vector. A statistically significant number of fluorescent cells was followed in "Time Lapse" to measure the migration speed and persistence. The YB-1 over-expressing cells showed an increase in average speed of migration by a factor of 1.3 and a reduction in the persistence of 40% of control (cells transfected with the GFP expressing vector). In contrast, GFP-YB1 and ΔNp63α (+ dox) expressing cells behave almost comparable to the control. The obtained results indicate that p63 is able to reverse the motility phenotype induced by YB1 over-expression. Finally, thanks to a collaboration with Dr. Enzo Di Iorio of Eye Bank of Veneto, we examined the YB-1 and ΔNp63α expression in intact and regenerative (after an injury) corneal epithelium by immunohistochemistry. We observed the predominant p63 nuclear and YB-1 cytoplasmic localization. However, we have observed a frequent overlapping between YB-1 and p63 signals, particularly in those cells that move from the basal to the suprabasal layer of the corneal epithelium, where they lose the stem cell features and initiate terminal differentiation. Further studies will be needed to fully understand the role of the p63 and YB1 association in the regeneration of this tissue.

In conclusion, during my PhD work, I provided evidences of a ΔNp63α/YB-1 complex able to interfere with the PI3K/Akt signalling pathway. I also proved that ΔNp63α can affect the ability of YB-1 to support the epithelial-mesenchymal transition. Therefore, my work provides additional knowledge on pathways that are crucial for tumor development and lays the premises for the study of new molecules and therapeutic strategies to counteract tumor pathogenesis and/or progression.

Abbreviations

AEC Ankiloblpharon-ectodermal dysplasia–clefting

ChIP Chromatin ImmunoPrecipitation

CSD Cold-Shock Domain

DBD DNA-binding domain

ED Ectodermal Dysplasia

EEC Ectrodactyly-ectodermal dysplasia–clefting syndrome

eIF eukaryotic Initiation Factor

EMT Epithelial-Mesenchymal Transition

GBM Glioblastoma Multiforme

GFP Green Fluorescent Protein

HNSCC Head and Neck Squamous Cell Carcinoma

LMS Limb mammary syndrome

OD Oligomerization domain

PIK3CA Phosphoinositide-3-kinase Catalytic Alpha polypeptide.

RNP Ribonucleoprotein

SAM Sterile Alpha Motif

SCC Squamous Cell Carcinomas

SHFM Split Hand Split Foot malformation

siRNA small interfering RNA

TA Transactivation domain

TGF Tumour Growth Factor

TID Transactivation inhibitory domain

YB-1 Y Box Binding protein 1

Introduction

1.1 The p63 gene discovery

The p53 tumor suppressor gene that plays an unprecedented role in human cancer was, for a long time, thought to be unique (in structure and function) until the discovery of two additional members of this protein family: p73 in 1997 [Kaghad, M et al.1997] and p63 in 1998 [Yang, A.N. et al. 1998]. p63 and p73 were discovered by virtue of their homology with the p53 oncosuppressor, thus it was natural for researchers and clinicians to consider these newly discovered p53-like genes as tumor suppressors. Like p53, p63 and p73 use alternative promoters and multiple splicing events to generate an array of functionally and structurally distinct isoforms.
Phylogenetic analysis indicated that p63 and p73 are evolutionary older than p53, and it was suggested that p63 may be the evolutionary predecessor of both p53 and p73 [Yang, A.N. et al. 1998, Joerger, A.C. et al. 2009]. Non vertebrate animals have only one gene of the p53 family, and it has a high degree of similarity to vertebrate ΔNp63 [Stifanic, M et al. 2009]. Interestingly, while the evolutionarily older family members, p63 and p73, play crucial but different roles in mammalian ontogenesis, p53 is specifically responsible for tumor suppression. However, p53, p63 and p73 share a common protein structure, which arises from their function as transcription factors. They consist of a central DNA-binding domain (DBD), a transactivation (TA) domain, and an oligomerization domain (OD). Furthermore, all three act as tetramers, and partial homology in the OD results in their potential ability to form heterotetramers. The central DBD domain of p63 is highly homologous with the DBDs of p53 and especially p73, suggesting that the three members of the p53 protein family can potentially share the ability to transactivate a common set of target genes [Yang, A.N. et al. 1998]. However, increasing evidences indicate that each homolog can exhibits specificity for the control of a particular set of genes [Dohn, M. et al. 2001 - Vigano, M.A et al. 2006].

1.1.2 TP63 gene and the p63 protein isoforms

The human TP63 gene maps on chromosome 3q28 and it consists of 15 exons and two alternative promoters (Fig. 1) (Yang, A. et al., 1998).
P63 protein isoforms include full-length isoforms having a transactivation (TA-) domain homologous to that of full-length p53, and amino-terminally truncated (ΔN-) isoforms. Alternative promoter usage and multiple splicing events contributes to the enormous complexity of p63 gene expression (Fig. 1) [Mangiulli et al., 2009].Transcription from the first promoter, located upstream of exon 1, gives rise to the full-length protein TAp63. Transcription from the second promoter results in the production of N-terminally truncated protein isoforms, ΔNp63. Alternative splicing, at the 3' end of TP63 mRNA generates distinct C-terminal protein isoforms that are known as α, β, γ and the newly described δ and ε.
The TA and ΔNp63α protein isoforms contain a conserved Sterile Alpha Motif (SAM), also present in proteins controlling development and cell differentiation processes. The SAM domain is known to be responsible for protein-protein interactions [Thanos, C.D. and Bowie, 1999]. Alpha isoforms also have a C-terminal transactivation inhibitory domain (TID). The TID domain is able to physically bind and negatively regulate TAp63α transcriptional activity through an intramolecular inhibitory mechanism [Ghioni et al. 2002, Helton et al. 2006]. Accordingly, the splicing isoforms lacking this region (β, γ) have stronger transactivation potential. Interestingly, The TID domain is known to be removed by activated caspases following pro-apoptotic stimuli [Sayan et al. 2007]. [Serber et al. 2002].
ΔNp63 isoforms that do not contain the N-terminal TA domain act as dominant negative of TAp63 isoforms and other transcriptionally active members of the p53 family (TAp73 and p53), partly due to its competition for binding to promoters of the target genes and partly due to the formation of non-functional hetero-oligomers. According to this model, the balance between the TA and ΔN isotypes, and the splicing isoforms (β, γ, lacking the TI) can finely regulate p63 functional activity.
The observation that ΔNp63α enforced expression is able to transactivate p53 responsive genes and induce cell-cycle arrest and apoptosis was truly unexpected [Dohn, M. et al. 2001]. However, more recently, a second TA domain (TA2), located downstream the DNA binding domain, has been identified and

described to confer an intrinsic transcriptional activity to ΔNp63 isoforms [Ghioni et al. 2002].

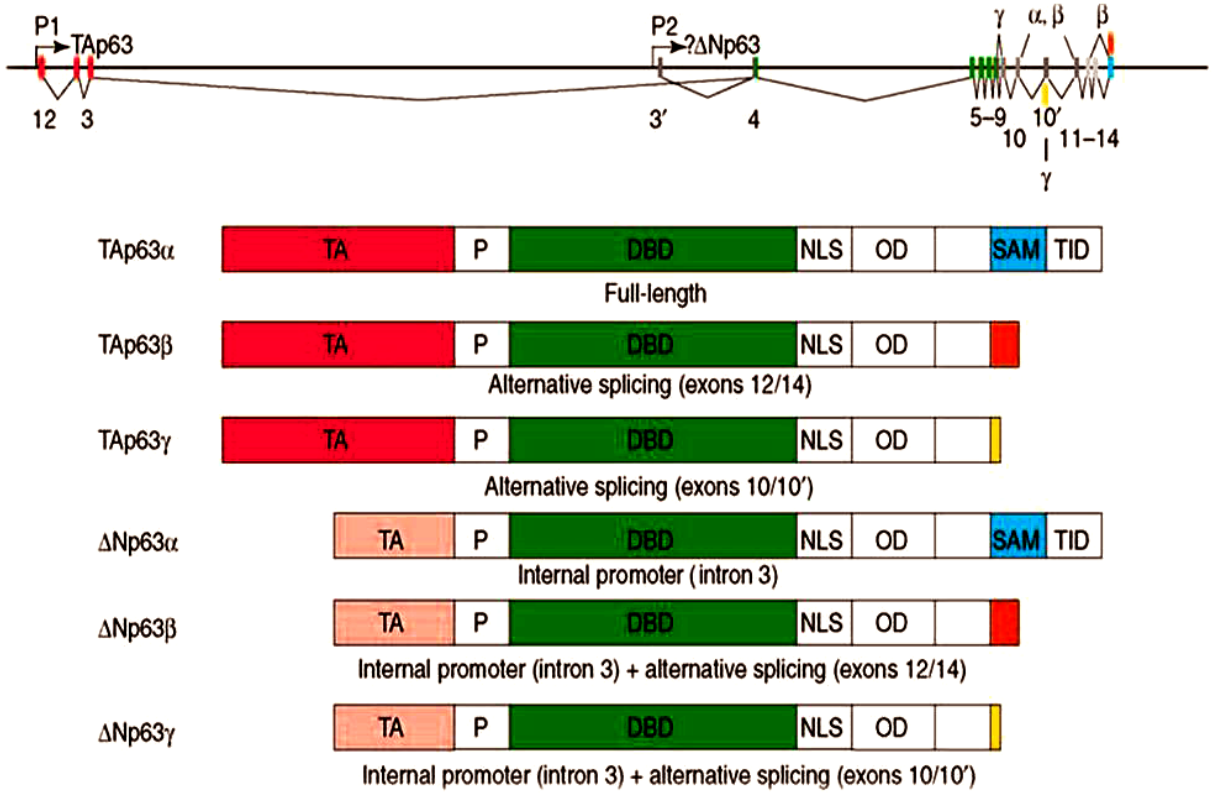


Figura 1: Transcription from the upstream promoter, P1, generates TAp63 isoforms that contain a TA domain homologous to that of full-length p53 (red). Transcription from the internal promoter, P2, generates the N-terminally truncated isoform class ΔNp63. Although ΔNp63 is devoid of the p53-like TA domain present in the TAp63 isoforms these proteins have a unique TA domain (red hatched bars) encoded by exon 30. Alternative splicing of C-terminal exons generates a isoforms containing a sterile alpha motif (SAM domain, blue) followed by a transcriptional inhibitory domain (TID). β and γ isoforms have unique C-termini (orange and yellow, respectively). Abbreviations: DBD, DNA binding domain (green); NLS, nuclear localization sequence; P, proline-rich domain; OD, oligomerization domain.

1.1.3 Functional role of p63 in the control of cell proliferation transformation and death.

The p63 gene encodes for products displaying similar or antagonistic activities. For instance TAp63 isoforms, and particularly the gamma isoform, are similar to p53 with regards to its ability to modulate genes that mediate cell cycle arrest and apoptosis— including BAX, p21, PUMA (p53-upregulated modulator of apoptosis).

On the other hand, consistent with its proposed oncogenic role, ΔNp63α was shown to repress the pro-apoptotic gene IGFBP-3 and transactivate HSP70, an anti-apoptotic stress-response protein associated with cell malignancy and transformation that play protective roles against cell death induced by a variety of insults – an observation that contrasts with the ability of ΔNp63 to induce apoptosis [Wu G. et al. 2005]. [Barbieri et al. 2005]. ΔNp63 can be degraded by p53 upon DNA damage, resulting in p53-dependent activation of glycogen synthase kinase (GSK)-3b followed by b-catenin degradation [Ratovistski, E. et al. 2001]. However, in epidermal cancers, ΔNp63 is not degraded by mutant p53, and is free to bind to the B56α subunit of protein phosphatase 2A and alter its conformation. This results in a dramatic inhibition of GSK3β with dismantling of the APC complex: the non-phosphorylated β-catenin aberrantly accumulates in the nucleus, resulting in the activation of T-cell factor and lymphocyte enhancer binding factor responsive elements, which contributes to tumourigenesis [Patturajan, M. et al. 2002].

Because of the ability of p63 to bind p53-like consensus sites, several attempts have been made to characterize p63 specific target genes. For instance, p63 has been shown to activate many – but not all – p53 responsive genes, such as p21, Bax and mdm2, although less efficiently. By microarray analysis, it was identified a set of genes selectively activated following infection of Saos2 cells with recombinant adenoviruses expressing TAp63α and ΔNp63α [Wu, G. et al. 2003]. Not unexpectedly, genes modulated by both TAp63α and ΔNp63α include those involved in cell-cycle control, cell signalling, proliferation and apoptosis, and epidermal differentiation. Again, many of those genes induced by TAp63α were also p53-responsive, such as p21, c-fos and IGFBP-3. However, TAp63a failed to activate many p53-responsive targets and also influenced the expression of many genes unaffected by p53. This suggests that p53 and TAp63 isoforms have overlapping but distinct functions within the cells.

TAp63 was shown induce apoptosis through two distinct mechanisms: (1) activation of death receptors and (2) the mitochondrial pathway. Actually, TAp63α can trigger the expression of CD95 , TNF-R and TRAIL-R death receptors. Furthermore, TAp63α upregulates the expression of proapoptotic Bcl-2 family members like Bax and BCL2L11 and the expression of RAD9, DAP3 and APAF1. Of clinical relevance is the fact that TAp63α is induced by many chemotherapeutic drugs and that inhibiting TAp63 function leads to chemoresistance [Gressner O et al. 2005]. On the

other hand, in p53 null human breast cancer cells, unlike TAp73, TAp63 cannot functionally replace p53 in adriamycin-induced cell cycle arrest and apoptosis thus raising some reasonable doubts on the relevance of p63 as a pro-apoptotic gene [Vayssade M, et al. 2005].
Flores ER and collaborators [Nature 2002] demonstrate that p63 is required for p53-mediated apoptosis in E1A-expressing mouse embryonic fibroblasts; whether this effect is specifically due to E1A expression, or not, still needs to be clarified. Nonetheless, it was demonstrated that p63 was required for radiation-induced p53-mediated apoptosis in developing nervous system, a more physiological setting. Finally, an *in vivo* study demonstrated that p63 is dispensable for both p53-dependent and p53-independent apoptosis in T-cells, in response to both gamma irradiation and receptor-mediated T-cell activation [Senoo M et al 2004; Benchimol S 2004]. All together, the available experimental evidences seem to indicate that p63 can potentially play a pro-apoptotic role but the relevance of p63 activity in the induction and control of apoptosis depend on the particular stimuli and cell contexts.

1.1.4 Role of p63 in Skin and Epithelial Development

The central role of p63 in epidermal development became established with the publication of the two independent studies on p63 -/- mouse models, generated by disrupting exons common to both TA and ΔN isoforms [Yang et al. 1999; Mills et al. 1999]. Both mice were born alive, but died within hours after birth because they fail to develop skin.
The p53 knock-out mice exhibited high incidence of tumors but no developmental abnormalities. Two independent groups generated p63-deficient mouse models, and these mice had severe developmental abnormalities that precluded the analysis on whether germ line p63-knockout causes a tumor-prone phenotype [Yang A, et al. ; Mills et al. 1999].
Given that also p53+/- heterozygous mice develop highly penetrant tumors, although with a delayed onset in comparison with that of p53 null mice, it seemed straightforward to ask whether p63+/- mice were also tumor prone.
Flores and collaborators [Flores ER, et al. 2005] reported that, although they did not display a highly penetrant tumor phenotype, p63+/- mice were prone to tumor development more than the wild type mice and in their tumor cells the wild type p63 allele was lost.They also observed that p63 heterozygosity in a

p53-compromised background increased tumor burden and enhances metastasis, supporting the hypothesis that p63 cooperates with p53 in tumor suppression, *in vivo*.
An independent study by Alea Mills and MW Keyes reached exactly the opposite conclusion showing that p63+/- mice are not prone to either spontaneous or chemically induced tumors. In addition, they found that p63 heterozygosity effectively reduced spontaneous tumor incidence in p53-compromised mice, indicating not only that p63 is not working as a tumor suppressor but that reduced dosage of p63 might even be protective against tumor [A. Mills et al. 1999]. This hypothesis is also supported by the observation that p63 deficiency in adult mice activates a program of cellular senescence [Keyes WM et al. 2005] an irreversible, cell cycle arrest that effectively halts aberrantly proliferating cells [Sharpless NE, DePinho RA 2005]. The take-home message of the Flores study [Flores ER, et al. 2005] is that p63 functions as a tumor suppressor, and that combined loss of p63 and p53 cooperates in malignancy, whereas the Keyes study concludes that p63 does not function as a tumor suppressor and supports the hypothesis that reduced p63 decreases the tumor-prone phenotype of p53-compromised mice [WM Keyes and AA Mills, unpublished]. The different conclusions might result from the fact that the two studies used mice heterozygous for distinct p63 alleles, one of which retains the reading frame in the targeted allele[Yang A, et al. ; Mills et al. 1999]. Indeed, initial reports of the phenotypes caused by p63 deficiency led to different models for the role of p63 in skin morphogenesis [McKeon F 2004]. Do these different conclusions, which were based primarily on the presence or absence of epidermal markers of differentiation, reveal genetic differences between the distinct p63 alleles? The answer to this question might be also important for determining whether the two targeted p63 alleles function similarly in tumorigenesis.
The idea that reduced levels of p63 might protect the cell from malignancy is intriguing and in agreement with the supposed pro-proliferative role for p63. However, it appears quite clear that p63 and p53 are not equivalent in their ability to suppress uncontrolled cell proliferation although further experiments should be done to shed light on this controversial aspect of p63 biology.
ΔNp63 isoforms in the adult are strongly expressed in epithelial cells with high clonogenic and proliferative capacity, and p63-/- epithelial stem cells undergo premature proliferation rundown, both in epidermis and thymus, indicating that p63 is a key determinant of the proliferative capacity of epithelial stem cells [Senoo et al. 2007].

Two hypotheses for the role of p63 in epidermal development were proposed based on the phenotype of p63-/- mice, which are born with a single-layered surface epithelium instead of a fully stratified epidermis. In the first model, one round of epidermal stratification is predicted to occur, however, the epidermis would not be maintained due to a premature depletion of epidermal stem cells [Yang et al., 1999]. If this hypothesis is correct the epithelium would be expected to express K5 and K14. An alternative explanation is that p63 is involved in the commitment to stratification. In this model, epidermal stratification never initiate, therefore one would predict that this epithelium express K18, but not K5 and K14 [Mills et al., 1999]. Notably, the second hypothesis turned to be true as p63 null surface epithelial cells do not express K5 and K14, instead they express K18, a marker for the uncommitted surface ectoderm.

Additional evidences that p63 drives epidermal development were obtained by genetic complementation. By crossing TAp63α or ΔNp63α epidermal transgenic mice into p63-/- mice, Candi et al. generated animals expressing only one isoform under the K5 promoter to identify the relative contribution of each variant to the development of the epidermis and thymus [Candi et al. 2006; Candi et al. 2007]. Although they were unable to obtain a full reversion of the p63 null phenotype, the ΔNp63 complemented transgenic mice (p63-/-;ΔN) developed a significant epidermal basal layer, while those complemented with TAp63 (p63-/-;TA) are very similar to p63-/- mice. Double complemented mice, however, exhibited a greater degree of re-epithelialization with expression of keratins of the basal layer (K5 and K14) and later differentiation markers such as K1 and loricrin; however, complete cornification remained impaired [Candi et al 2006]. These data suggested that ΔNp63 is important for maintaining the proliferative potential of the basal layer, whereas TAp63 contributes to epidermal differentiation by acting synergistically with and/or subsequently to ΔNp63.

A study based on RNA interference directed against specific p63 isoforms, demonstrated that ΔNp63 isoforms are the main mediators of p63 effects while TAp63 isoforms contribute to later stages of differentiation, in mature keratinocytes. In this work, the roles of p63 in differentiation and cell proliferation were shown to be distinct. Indeed, downregulation of p63, in keratinocytes, causes a p53-dependent cell cycle arrest while simultaneous knockdown of p53 in the context of p63 loss is able to rescue cell proliferation but not the differentiation defect [Lee et al., 2002; Truong et al. 2006].

To get insights into the p63 functions, several approaches have been taken, over the last few years, to identify its target genes

These studies provided clear evidences that p63 controls and connects different pathways involving cell differentiation, adhesion and proliferation [Pozzi et al. 2009].
Though the original controversy on ΔNp63 being involved in stemness or differentiation has been solved, still the underlying molecular mechanisms involved await elucidation. Even though TAp63 expession is hard to detect in the epidermis, it seems to be induced under stress stimuli and during wound healing, indicating a role in tissue regeneration and cell death [Lin et al. 2009].

1.1.5 Human congenital diseases linked to p63 disfunction.

Given the role played by the p53-like proteins as master regulators of crucial developmental processes, the finding of pathological abnormalities linked to their dysfunction or imbalance, in humans, was expected. An extensive literature exists, supporting a crucial role for p63 in the pathogenesis of several human diseases.
Germ line mutations of p63 are found in humans and are responsible for several rare autosomal dominant developmental diseases, such as the Ectrodactyly-ectodermal dysplasia–clefting syndrome (EEC), the Ankiloblpharon-ectodermal dysplasia–clefting also known as Hay-Wells syndrome (AEC), the Limb mammary syndrome (LMS), the ADULT syndrome, the Rapp-Hodgkin syndrome and non syndromic Split Hand Split Foot malformation (SHFM). ED is clinically characterized by malformation of skin, hair, nails and teeth, and by facial clefts. In addition, EEC patients show lachrymal duct abnormalities, urogenital problems, hearing loss, facial dismorphism, respiratory infections, and developmental retardation [Celli et al. 1999]. Most commonly, p63 mutations give rise to either amino acid substitutions in the DBD that abolish p63 DNA-binding ability or to shifts of the reading frame, which specifically truncate the α-end region of p63 [Celli et al. 1999]. AEC patients have mutations giving rise to amino acid substitutions in the SAM domain [McGrath et al. 2001]. These missense mutations are predicted to disrupt protein–protein interactions, by either destroying the compact globular structure of the SAM domain, or substituting amino acids, which are crucial for such interactions. Further interpretation of the biological consequences of the SAM domain mutations is obscured by our lack of knowledge of the physiological role of this structural domain. Mutated proteins have been shown to act as dominant negative molecules and, in fact, the disease occurs at heterozygous level. Mutations in the p63 carboxy-

terminal-end cause SHFM. Several of these mutations lead to truncated forms of the protein that are shortened by eight (Q634X) or three (E639X) amino acids [Rinne et al. 2009]. These truncations remove a sumoylation site that seems to play an important role in the regulation of p63 [Ghioni et al. 2005; Huang et al. 2004; Straub et al. 2009].
The pattern of mutations has revealed p63 protein domains that are particularly important for skin development (the carboxy terminal SAM domain), whereas other domains are crucially important for limb development (DBD, carboxy-terminal-end domain). A striking genotype/phenotype correlation can be easily recognized [Van Bokhoven and Brunner 2002; Rinne et al. 2006; Rinne et al. 2007]. The underlying molecular mechanisms leading to these diseases are under active investigation.

1.1.6 p63 in cancer

The p63 gene is a tumor suppressor, an oncogene or both? The answer still remains an open question. Several studies supporting the hypothesis that p63 can function as a tumor suppressor rely on the ability of full-length TAp63 isoforms to trigger cell cycle arrest and apoptosis [Yang et al. 1998; Gressner, O. et al. 2005]. On the other hand, ΔNp63α is known to inhibit both death receptor-mediated and chemotherapy-induced apoptosis [Mundt, H.M., et al. 2010].
Increasing evidences support the idea that ΔNp63 actually promotes cancer development thereby functioning as an oncogene. Squamous cell carcinomas of the lung or head and neck are characterized by amplification of the p63 locus as well as overexpression of ΔNp63α [Yamaguchi, K et al. 2000-Tonon, G et al. 2005]. Moreover, ΔNp63α enforced expression was shown to enhance cell growth in soft agar and increase tumor size in mice [Hibi, K. et al. 2000].
However, the functional role of p63 isoforms in tumor pathogenesis and progression should not be studied without considering the effects due to other p53 family members. In fact it is still difficult to predict which might be the ultimate effect of hetero-tetramerization among p53-family members. For instance, even though the oligomerization domain (OD) of p53 does not associate with the homologous domain of p63 or p73, p63 and p73 isoforms can assembly by forming hetero-oligomers. ΔNp63 isoforms were described to affect the transactivation functions of TAp63, p53 and p73 via direct protein-protein interactions [Joerger, A.C et al. 2009].
Multiple mechanisms have been described to explain how p53 family members can regulate each-other's activity:

(1) First, mutant p53 was demonstrated to associate *in vitro* and *in vivo* with the p63 DNA binding domain and this interaction impairs the sequence-specific binding of p63. [Gaiddon, C et al. 2001, Strano, S et al. 2002]. The inactivation of p63 by mutant p53 might be one of the possible mechanisms conferring mutant p53 gain of function.This mechanism seems to be operative in T47D cells expressing a mutant p53: in these cells p63 is unable to recruit some of its target gene promoters [Strano, S et al. 2002].

(2) Secondly, the central domain of all p63 variants is highly homologous with the DBDs of p53 and p73, so that p63 can interact with the p53 consensus sites. TAp63 is able to transactivate reporter genes containing p53-responsive elements, while the ΔNp63 isoforms, lacking the N-terminal TA domain, can bind to p53 target sites in a competitive way and act as a dominant negative inhibitor of p53, TAp63 or TAp73. [Yang et al. 1998].

(3) Thirdly, a cross regulation between p53 family members has been demonstrated. p63 associates with its own promoter as well as with the promoter of p53 and p73. Therefore, p63 may regulate its own level of expression as well as that of its relatives. Remarkably, ΔNp63 transcription is regulated by p53 [Harmes, D.C. et al. 2003]. Disruption of p53 activity abolishes the expression of ΔNp63α. This regulation is mediated by a p53-binding element sufficient to confer these activities to a heterologous promoter. Chromatin immune-precipitation indicates that, in asynchronously growing cells, p53 occupies this element. In response to DNA damage, ΔNp63α is recruited to this element as transcription of deltaN-p63 declines. Disruption of deltaN-p63α expression had differential effects on the transcriptional regulation of several p53-target genes. These findings indicate that p53 contributes to the preservation of basal epithelia by driving the expression of deltaN-p63 isoforms. Moreover, ΔNp63 is recruited to and can activate its own promoter, thus providing an autoregulatory loop of self-regulation [Romano, R.A et al. 2006]. Importantly, loss of p53 leads to the stabilization of TAp63γ [Li, N. et al. 2006]. Consequently, disruption of TAp63γ expression leads to decreased expression of ΔNp63, and overexpression of TAp63γ enhances the activity of the ΔNp63 promoter. Thus, TAp63γ is capable of activating the expression of ΔNp63.

(4) Fourthly, p53 family members cooperate in cell cycle regulation and apoptosis. In MCF7 cells, p73 and p63, but not p53, are modulated during the cell cycle with a peak in S phase, and their silencing suppresses proliferation [Lefkimmiatis, K et al. 2009]. In cycling cells, p73 and p63 are bound to the p53-responsive elements (REs) in the regulatory regions of cell

cycle progression genes. However, when the cells are arrested in G0-G1, p73 detaches from the REs and is replaced by p53, which functions as transcriptional repressor. When the cells move into S-phase, p73 is recruited again and p53 is displaced or is weakly bound to the REs. Thus, elevated concentrations of p73 and p63, as it is found in many cancers, could cause the aberrant activation of cell growth progression genes and therefore contribute to cancer initiation or progression.

(5) The interaction among the p53 family members is also critical for the control of p73-dependent cisplatin sensitivity. In triple-negative primary breast cancers, that commonly exhibit mutational inactivation of p53, ΔNp63 and TAp73 isoforms are co-expressed. This subset of breast cancer exhibited cisplatin sensitivity that was dependent on TAp73 [Leong, C.O et al. 2007]. The ΔNp63α isoform promoted survival of breast cancer cells by binding TAp73, thereby inhibiting its pro-apoptotic activity. In response to treatment with cisplatin, but not other chemotherapeutic agents, TAp73 underwent c-Abl-dependent phosphorylation, which promoted dissociation of the ΔNp63α/TAp73 protein complex followed by TAp73-dependent transcription of Bcl-2 family members and apoptosis. Breast tumors with the above described intact pathway would be predicted to be platinum sensitive and have high levels of ΔNp63 to repress TAp73 activity. [Silver, D.P. et al. 2010]
Similarly, in HNSCC patients, high levels of ΔNp63 are associated with a good response to platinum-based chemotherapy [39]. In HNSCC cells, ΔNp63 inhibits the activity of TAp73 and suppresses TAp73-dependent apoptosis [Rocco, J.W et al. 2006]. TAp73 is necessary for apoptosis following knockdown of ΔNp63, and siRNA-mediated inhibition of TAp73 expression reduces HNSCC cellular sensitivity to cisplatin. Moreover, as shown by immunoprecipitation, ΔNp63 also forms a complex with TAp73 in HNSCC, therefore it is possible that a mechanism as that described in triple-negative breast cancer also takes place in HNSCC. These results should also be considered in relation to the effects of ΔNp63 in promoting cell survival, which behaves as a paradox to the cisplatin sensitivity of p63-expressing tumors. Indeed, in HNSCC, siRNA-mediated reduction in the endogenous p63 levels results in more tumor cells being killed by radiation and cisplatin, demonstrating a pro-survival role for p63 [Thurfjell, N et al. 2005]. These combined data indicate that the cooperative or antagonistic interactions between p53-family members are important for the regulation of the cell cycle and apoptosis, and thus also for tumor development. Moreover, there is substantial evidence that ΔNp63 expression is a predictor of chemosensitivity to cisplatin.

Ultimately, a tumor suppressor is a gene that is frequently lost or inactivated in human cancers. Based on this definition, however, drawing analogies between p63 and p53 is like “fitting a square peg into a round hole”. With regards to classical features of a tumor suppressor, p63 contrasts markedly with p53: it is rarely mutated in human cancers [Moll UM, Slade N 2004]. Indeed, the majority of tumors maintain p63 expression, and in many cases the p63 locus is amplified or p63 appears to be overexpressed, consistent with p63 performing a pro-proliferative or oncogenic role.
A genome-wide microarray screen of non-small cell lung cancer revealed that the 3q26–29 locus encompassing p63 is frequently amplified in squamous cell carcinomas of the lung, suggesting that overexpression of p63 facilitates tumorigenesis [Tonon G et al. 2005]. A study of wide number of esophageal tumor samples demonstrates that there was no correlation between p63 expression and p53 mutational status, thus suggesting that upregulation of p63 in squamous cell carcinomas is independent of p53. [Cui R, et al. 2005]. However, metastatic tumor types have been reported to lose p63 expression, thereby suggesting that loss of p63 might induce tumor metastases [Urist MJ et al. 2002, Koga F et al. 2003]. Whether more malignant tumors with undetectable p63 expression arose from progenitor cells that were expressing p63 or whether epithelial–mesenchymal transition, that frequently accompany tumor progression, occurs in p63 null tumors awaits further investigation.

1.1.7 p63 in cell adhesion, migration, and metastasis

An increasing number of data indicate that p63 is involved in cell migration and adhesion and thus also in processes connected with metastasis and wound healing. In HNSCC, p63 was shown to regulate the expression of adhesion-related genes and contribute to cell invasion and migration [Gu, X.L et al. 2008]. In squamous cell carcinoma disruption of p63 was found to trigger the expression of genes associated with a higher potential to metastasize and invade [Barbieri, C.E et al. 2006]. Additionally, *in vitro* cell migration assays showed that loss of p63 leads to increased cell migration of squamous carcinoma cells[Barbieri, C.E et al. 2006].
In a recent manuscript, Adorno et al. (2009) described that in cells expressing mutant p53, p63 acts as an antagonist of TGFβ-mediated tumor invasiveness and metastasis. Specifically, p63 transcriptional activity was found to be

inactivated by a mutant p53-Smad complex induced by TGFβ. [Adorno, M et al. 2009].
Similarly, knockdown of p63 expression caused down-regulation of cell adhesion associated genes, cell detachment and anoikis in mammary epithelial cells and keratinocytes [Carroll, D.K et al. 2009]. Most recently, Su et al. showed that TAp63 suppresses metastasis by regulating the microRNA processing complex [Su, X et al. 2010]. These findings uncovered a new role of p63 as a negative regulator of metastasis. Bamberger et al. suggested the role of p63 isoforms in the healing of skin wounds, which is achieved by extensive migration and hyperproliferation of keratinocytes [Bamberger, C et al. 2005]. ΔNp63 variants were found at high levels in basal and suprabasal keratinocytes of the hyperproliferative wound epithelium, TAp63 variants were also expressed in wound keratinocytes. Thurfjell et al. compared the p63 status in normal oral wounds and in HNSCC, two situations that represent self-limiting and non-self-limiting processes[Thurfjell, N et al. 2006]. They found that both processes require upregulation of TA and ΔNp63α. However, in wounds, there was a down-regulation in TAp63 mRNA levels but not in protein expression, indicating a post translational mechanism of TAp63 stabilization. In conclusion, a finely tuned balance between of p63 isoform transcription and protein stabilization appears to be required in order to drive normal epithelial proliferation and differentiation.

1.2 YB-1: a newly identified p63 partner

Unequivocal establishment of the biological role of p63 is complicated by the fact that this protein exists in multiple isoforms with sometimes seemingly contradictory activities. Furthermore, the activity of each specific isoform can be deeply modified or modulated through the dynamic interaction with particular molecular partners. In the laboratory where I carried out the work for my PhD thesis, a total of 50 potential ΔNp63α interacting proteins were isolated by a functional proteomic approach [Amoresano et al., 2010]. Several RNA/DNA binding proteins were identified including the YB-1 oncoprotein. The finding that ΔNp63α is engaged in complexes with YB-1 laid the premises for my work aimed to understand the functional role of their association in the control of cell proliferation and trasformation.

1.2.1 YB-1 STRUCTURAL ORGANIZATION

Mammalian Y Box Binding protein 1 (YB-1) is a 36 kDa protein showing abnormal electrophoretic mobility with an apparent molecular weight of 50-kDa. The protein is enriched in Arg, Pro, Gly, and Gln and exhibits a pI of about 9.5. Three structural domains can be distinguished in YB-1 (Fig. 2): a small N-terminal domain rich in Ala and Pro (A/P domain), a central cold-shock domain (CSD), and a C-terminal domain (C domain) with alternating clusters of positively and negatively charged amino acid residues (four clusters of each sign) [Matsumoto K., Wolffe A.P. 1998; Sommerville J. 1999]. The CSD is a highly evolutionarily conserved domain.

In bacteria, proteins consisting of the CSD domain alone are known as the major cold-shock proteins: synthesis of some of these proteins is activated at low temperature and facilitates adaptation of bacteria to cold conditions [Graumann P.L., Marahiel M.A. 1998]. Bacterial major cold-shock proteins and the CSD of YB-1 have the same β-barrel spatial structure [Schindelin H et al. 1993; Kloks C.P et al. 2002]. The spatial structures of the N and C domains of YB-1 are still unknown. However, it is generally believed that the C domain assumes various conformations depending on its interaction partner [Manival X. Et al. 2001; Ivanyi-Nagy R et al. 2005]. Like the bacterial main cold-shock proteins, eukaryotic CSD harbors conserved motifs RNP1 and RNP2 and interacts both with DNA and with RNA [Kohno K et al. 2003; Sommerville J. 1999; Graumann P.L., Marahiel M.A. 1998]. The C domain has a nonspecific affinity for RNA and DNA and, presumably, utilizes its positively charged clusters to interact with negatively charged phosphate groups of nucleic acids. Isolated YB-1 forms large homo-multimeric complexes, with a molecular weight reaching 800 kDa and a sedimentation coefficient reaching 20S [Evdokimova V.M et al. 1995, Gaudreault I et al. 2004]. In electron and atomic force microscopy on a substrate, such multimers are seen as rounded uniform structures of 30–40 nm in diameter and 8–10 nm in height [Murray M.T et al. 2004]. The protein is multimerized probably as a result of interactions between differently charged C domain clusters. The interaction of YB-1 with other proteins can involve each of the three domains alone or their combinations [Kohno K et al. 2003, Sorokin A.V et al. 2005].

YB-1 belongs to a large family of proteins possessing CSD. The embryonic proteins accumulate to a high percentage in gametes and play a role in packaging and masking mRNA in the cytoplasm [Matsumoto K., Wolffe A.P. 1998; Sommerville J. 1999]. Compared with YB-1 these proteins have not only CSD

with a fully identical amino acid sequence, but also a similarly organized C domain.
For instance, Xenopus embryonic protein FRGY2 is similar to somatic protein FRGY1 in having the same number of positive and negative amino acid clusters in the C domain [Murray M.T et al. 1992; Wolffe A.P.et al. 1992]. The embryonic and somatic proteins are near identical in pI and the amino acid composition. At the same time, beyond CSD the amino acid sequence identity of the two proteins is below 30%. FRGY2 is dephosphorylated upon fertilization of Xenopus eggs, which, possibly, decreases its affinity for RNA and provides a mechanism of mRNA demasking [Sommerville J. 1990; Sommerville J., Ladomery M. 1996]. Embryonic FRGY2 is replaced by somatic FRGY1 during further embryo development; the replacement is completed by the end of gastrulation [Wolffe A.P et al. 1992, Tafuri S.R., Wolffe A.P. 1990].
In Xenopus oocytes the YB-1 protein exists in two isoforms, known as p54 and p56 [Murray M.T., Schiller D.L., Franke W.W. 1992]. Chironomus also has two isoforms, Ct-p40/p50, that differ only in the C-terminal amino acid sequence and, presumably, are encoded by two mRNAs produced from one primary transcript via alternative splicing [Soop T et al. 2003]. Both Ct-p40 and p50 were found in the larval salivary glands, while only one isoform, Ct-p40, occurs in cultured embryonic cells. Other proteins related to YB-1 have greater structural differences and in some cases similarity is restricted to the presence of CSD [Matsumoto K., Wolffe A.P. 1998, Sommerville J. 1999].

1.2.2 YB-1 IS A MULTIFUNCTIONAL DNA/RNA-BINDING PROTEIN

Mammalian YB-1 regulates various activities of cells and the whole organism, including cell growth, differentiation, stress response, and programmed cell death (apoptosis) [Skabkin M.A et al. 2004]. YB-1 is necessary for cell adaptation to growth at suboptimal temperatures [Matsumoto K. et al. 2005]. YB-1 increases cell resistance to ionizing radiation and xenobiotics, and its transfer into the cell nucleus is regarded as the earliest marker of multiple drug resistance in cancer cells [Kohno K et al. 2003, Kuwano M et al. 2004]. The YB-1 content in some tumors (breast cancer, lung cancer, carcinoma of the prostate, osteosarcoma, etc.) is higher than in normal cells of the same tissue. Moreover, the YB-1 concentration in non-small-cell lung cancer is so high that YB-1 has been included in the list of the 12 proteins whose amounts changed most significantly upon

cell malignant transformation. Enforced expression of YB-1, causes centrosome amplification and leads to numerical chromosome instability and oncogenic transformation of cells [Bergmann S et al. 2005]. In other words, YB-1 can be regarded as a tumor marker.

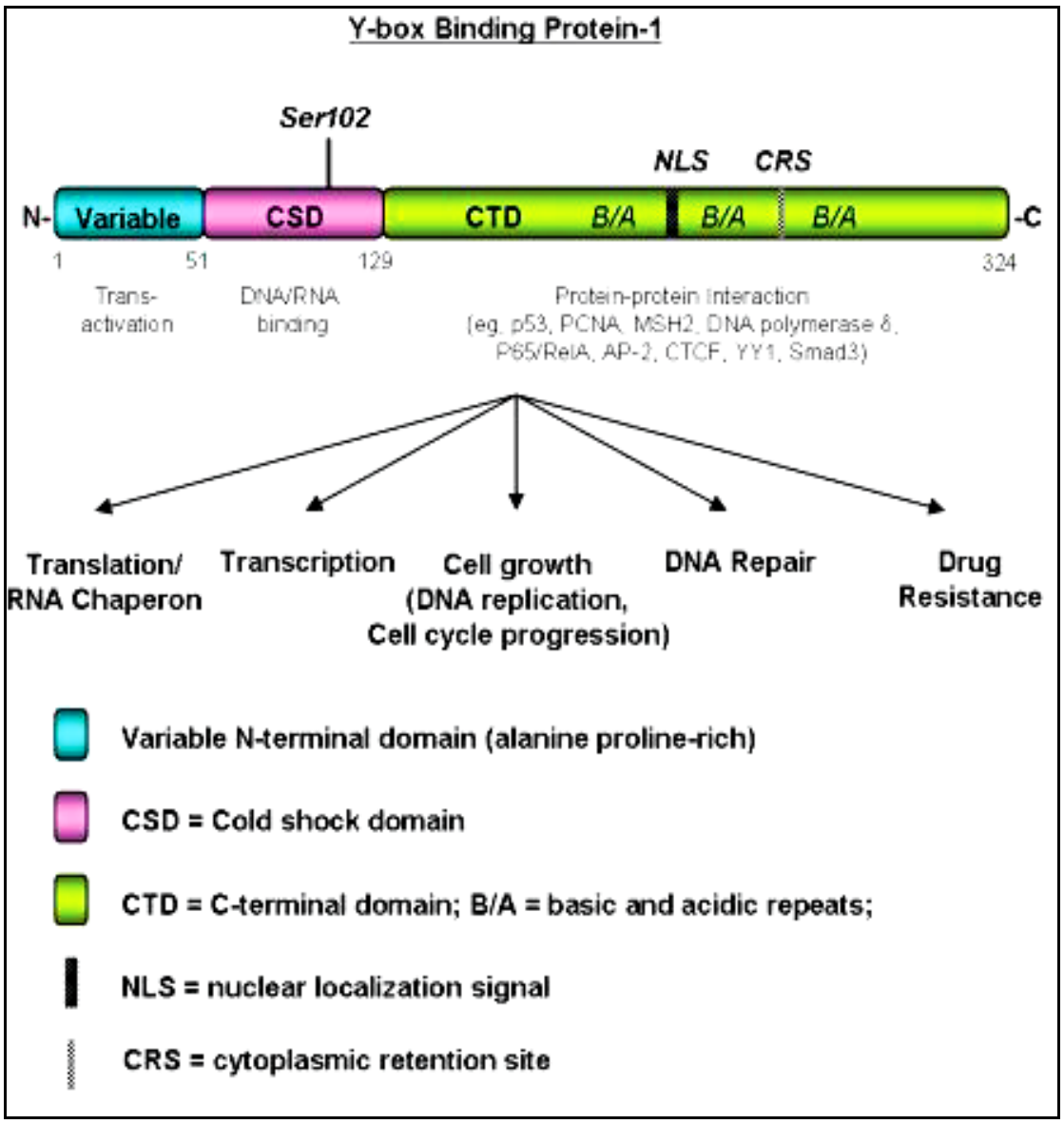


Figura 2 The Structure and Functions of YB-1. YB-1 is made up of the N-terminal, cold shock (CSD) and C-terminal domains (CTD). These domains have unique functions. The N-terminal is necessary for transactivation whereas the CSD is important for RNA/DNA binding. Most of the characterized protein-protein interactions occur on the CTD. The CSD and CTD also work together to facilitate nuclear trafficking. Cellular trafficking is furthermore regulated by the nuclear localization signal in the C-terminal domain as well as the cytoplasmic retention signal also located in this region of the protein

Although acting as an oncoprotein in many cases, YB-1 can also prevent oncogenic transformation of cells via the inositol 3-phosphate/ Akt-kinase signaling pathway [Bader A.G et al. 2003]. Suppression of YB-1 synthesis in knock-out mice caused postnatal death, and exhibit exencephaly associated with

defective neural tube closure and abnormal patterns of cell proliferation within the neuroepithelium. [Lu Z.H. et al. 2005].
One of the key function of YB-1 consists in the relatively unique ability of this protein to interact with both DNA and RNA [Kohno K et al. 2003, Matsumoto K., Wolffe A.P. 1998]. YB-1 has a general high affinity for any nucleotide sequence in DNA or RNA, although, in some conditions, particular sequences are bound preferentially [Minich W.B et al. 1993– Coles L.S et al. 2004]. YB-1 specifically binds to certain regulatory DNA sequences, in particular, promoter and enhancer regions containing Y boxes (inverted CCAAT motifs in a special surrounding).
YB-1 regulates, positively or negatively, transcription of genes involved in relevant cellular pathways [Kohno K.,et al. 2003 Swamynathan S.K et al. 1998. These include growth factor genes, division genes , apoptosis, in the immune response, multiple drug resistance, stress responses and some viral genes.
YB-1 displays a higher affinity for single stranded regions of DNA and RNA, and substantially decreases the melting temperature of double helices [Zasedateleva O.A et al. 2002, Evdokimova V.M et al. 1995– Izumi H. Et al. 2001]. In fact, *in vitro* experiments have shown that YB-1 is able to accelerate the annealing of complementary strands of nucleic acids, by three orders of magnitude. Under physiological conditions, it favours the formation of the longest and most perfect duplexes [Skabkin M.A et al. 2001].
The only exception is DNA double helices with poly(G) blocks, whose melting temperature is significantly increased by YB-1 [Zasedateleva O.A et al. 2001].
YB-1 has a higher affinity for DNA regions with a damaged secondary structure and promotes DNA repair. Accelerating the exchange of complementary nucleotide strands in double helices, YB-1 presumably contributes to DNA recombination [Skabkin M.A et al. 2001, Ise T et al. 1999]. There is evidence that YB-1 plays a role in DNA replication [Levenson V.V et al. 2000, En-Nia A et al. 2005]. Thus, YB-1 is probably involved in all DNA-dependent processes. During transcription YB-1 binds to pre-mRNA on chromosomes and accompanies mRNA molecules throughout their life [Kohno K et al. 2003, Soop T et al. 2003].
In the cell nucleus, YB-1 plays a role in alternative splicing of pre-mRNA [Stickeler E. Et al. 2001, Chansky H.A. et al. 2001]. In the cytoplasm of mammalian cells, almost all YB-1 molecules are associated with translated or non translated mRNAs, determining their functional activity [Davydova E.K. et al. 1997, Evdokimova V.M., Ovchinnikov L.P. 1999], stability

[Evdokimova V et al. 2001], and the localization of translationally active mRNAs on the actin skeleton [Ruzanov P.V et al. 1999]. It is clear that YB-1 is involved not only in DNA dependent processes, but in all steps of mRNA biogenesis and functioning as well. The various effects of YB-1 on mRNA-dependent processes, including global regulation of protein synthesis, are due to its relatively non specific interactions with mRNA [Ovchinnikov L.P. et al. 2001]. At the same time, the specific affinity of YB-1 can serve to selectively regulate synthesis of certain proteins, including YB-1 itself [Coles L.S. et al. 2004, Giorgini F et al. 2001, Skabkina O.V. et al. 2005].

The involvement of YB-1 in particular cell processes depends on its distribution between the nucleus and the cytoplasm. YB-1 is mostly located in the cytoplasm [Jurchott K. et al. 2003, Sorokin A.V. et al. 2005]. However, YB-1 is transferred into the cell nucleus for a relatively short period at the G1/S transition during the cell cycle [Jurchott K. et al. 2003], activating transcription of the cyclin A and B1 genes. The transfer of YB-1 into the nucleus has been observed in response to UV light and DNA-damaging chemicals [Koike K et al. 1997, Gaudreault I et al. 2004]. In addition, YB-1 is transferred into the nuclei of cultured vascular endothelial cells in response to thrombin and, acting as a transcription factor, activates the expression of genes involved in endothelial cell differentiation [Stenina O.I et al. 2001].

YB-1 contains the nuclear localization and cytoplasmic retention signals (Fig. 2) [Jurchott K et al. 2001, Stenina O.I et al. 2001, Bader A.G., Vogt P.K. 2005]. The cytoplasmic retention signal is in the C-terminal portion of the molecule. Both full-length YB-1 and its form truncated at the C end have been found in the cell nucleus [Sorokin A.V et al. 2005]. The mechanism of transferring full length YB-1 into the nucleus is as of yet unclear. In endothelial cells treated with thrombin and in NIH3T3 cells exposed to genotoxic stress upon treatment with doxorubicin YB-1 is transferred into the nucleus owing to the cleavage of its C-terminal region containing the cytoplasmic retention signal [Sorokin A.V et al. 2005, Stenina O.I et al. 2001]. The cleavage is catalyzed by the proteasome, because the proteasome inhibitor MG132 prevents both the C cleavage of YB-1 and its transfer into the nucleus [Sorokin A.V et al. 2005]. The selective proteasomal C-cleavage of YB-1 has been reproduced in vitro with the 20S core proteasome and has been shown to involve neither ATP nor ubiquitination of YB-1. The protein is cleaved only when non bound to RNA. It is thought that YB-1 can enter the 20S proteasome channel with catalytical activities because its C-terminal region is unstructured [Sorokin A.V et al. 2005].

C-truncated YB-1 retains the capability of activating transcription of certain genes [Stenina O.I et al. 2001].

1.2.3 NONSPECIFIC INTERACTION OF YB-1 WITH mRNA

YB-1 was identified, more than 30 years ago, as a universal major protein of cytoplasmic mRNPs [Morel C. et al. 1971; Blobel G. 1972]. The protein, known as p50 at that time, was detected in mRNP preparations from various cells and organisms [Preobrazhensky A.A., Spirin A.S. 1978; Dreyfuss G. 1986]. YB-1 displays an extremely high affinity for mRNP (Kd ~ 4 nM for the globin mRNA) and approximately the same affinity for the bacterial 16S rRNA in vitro [Minich W.B et al. 1993], suggesting a high affinity for a great diversity of nucleotide sequences. There is evidence that the efficiency of binding with YB-1 sharply drops when the size of RNA is below a certain threshold (about 50 nucleotides) [Bouvet P et al. 1995, Jiang W. et al.1997].
YB-1 protects RNA from chemical agents. The aspecific binding of YB1 to mRNA occurs with the nucleic-acid sugar-phosphate backbone [Pisarev A.V. et al. 2002]. YB-1 utilizes both the CSD and the C-terminal domain to interact with RNA [Izumi H. et al. 2001, Pisarev A.V. et al. 2002]. The binding of CSD to RNA probably involves positively charged and aromatic residues of RNP1 and RNP2, while the C-terminal domain seems to bind with clusters of positively charged residues [Blobel G. 1973]. However, covalent cross-linking experiments, performed to study the interaction of YB-1 with the mRNA 5'cap, revealed that the CSD but not the C domain, of YB-1 displaces the eIF4E initiation factor from its complex with the 5'cap of mRNA and that mutations affecting the aromatic residues of RNP1 completely abrogated its interaction with the cap [Pisarev A.V. et al. 2002].
The free non translated mRNPs contains only YB-1 as the major core protein. In translated polysomal mRNPs, instead, YB-1 is present with the poly(A)-binding protein (PABP) [Blobel G. 1973; Minich W.B., Ovchinnikov L.P. 1992]. The YB-1 content in polysomal mRNP is half as high as in free mRNP [Minich W.B et al. 1993]. This indicates that, when mRNA changes from the non-translated to the translated state, a large amount of YB-1 dissociates from it and PABP binds to the poly(A) tail. YB-1 binds different homopolyribonucleotides with different affinities [Minich W.B et al. 1993]. The affinity decreases in the following order: poly(G) > poly(U) > poly(A) > poly(C). The affinity of YB-1 for poly(A) is comparable with the

affinity of PABP for the same sequence. Thus, the two major proteins of mRNP can compete with each other for binding to the poly(A) tail of mRNA.
YB-1 exerts a dual effect on translation, stimulating it at a low YB-1/mRNA ratio, while completely suppressing it at a higher YB-1/mRNA ratio [Minich W.B., Ovchinnikov L.P. 1992]. Maximal stimulation of protein synthesis was observed at the YB-1/mRNA ratio characteristic of polysomal mRNP. Translation is completely suppressed when the YB-1/mRNA ratio is close to that in free non-translated mRNP. Stimulation and suppression of translation by YB-1 occur only at initiation [Evdokimova V.M et al. 1998; Nekrasov M.P et al. 2003]. To stimulate initiation YB-1 most likely promotes the scanning of the 5'-untranslated region (5'-UTR) by the 43S preinitiation complex searching for the initiation triplet. YB-1 suppression of translational start leads to the accumulation of mRNA in the form of free mRNP.
This mechanism of suppression is only partly explained by PABP displacement from its mRNA complex, as YB-1 inhibits not only translation of poly(A)+/mRNAs, but also that of poly(A)-/mRNAs.
YB-1 blocks the interaction of mRNA with initiation factors of translation, primarily eIF4G [Nekrasov M.P et al. 2003]. In addition YB-1 stabilizes mRNA both *in vitro* and *in vivo* by a mechanism requiring the 5'-terminal cap [Evdokimova V. Et al. 2001, Chen C.Y. et al. 2000]. It is of interest that different domains of YB-1 are responsible for inhibition of translation and mRNA stabilization [Evdokimova V. Et al. 2001; Nekrasov M.P et al. 2003]. Stabilization of mRNA is due to the YB-1 fragment containing CSD and the A/P domain while the translation inhibitory activity belongs mostly to the C domain. These two fragments displace different subunits of eIF4F from its complex with mRNA: AP-CSD displaces the cap-binding subunit eIF4E, while the C domain displaces the eIF4G subunit.

1.2.4 SPECIFIC INTERACTION OF YB-1 WITH mRNA

The regulation of protein synthesis is driven by particular proteins or RNAs that interact with 3'- or 5'-UTR mRNA sequences [Wilkie G.S. et al. 2003]. Recently, a regulatory 3'-UTR sequence was found in YB-1 mRNA [Skabkina O.V. et al. 2003]. A short RNA fragment containing this sequence strongly suppressed YB-1 synthesis when added to a cell-free translation system. Further studies demonstrated that the fragment inhibited translation of YB-1 mRNA as well as that

other transcripts. UV-inducible crosslinking experiments revealed that YB-1 and PABP proteins specifically bind to this fragment [Skabkina O.V. et al. 2003]. The specific binding of PABP to exogenous poly(A) inhibited translation of YB-1 mRNA and was accompanied by the accumulation of mRNA in the form of free mRNP [Skabkina O.V. et al. 2003]. Inhibition was observed even when the YB-1 mRNA was devoid of the poly(A) tail. [Skabkina O.V. et al. 2005].
As in the case of depletion of PABP, free mRNP accumulated as a result of such a suppression. Thus, initiation was suppressed at the step of mRNA binding to the 43S preinitiation complex, or at an earlier step of mRNA interaction with the translation initiation factors in both cases.
The specific binding of YB-1 to certain mRNAs affects both nuclear and cytoplasmic processes. Several examples are known for the role of Y-box binding proteins in selective mRNA stabilization. For instance, (1) YB-1 binds to an A/C-rich region of exon 4 of the CD44 pre-mRNA thereby increasing the rate of exon incorporation into the mature mRNA [Chansky H.A. et al. 2001], (2) The specific binding of Chk-YB-1 to the Rous sarcoma virus RNA suppressed synthesis of viral p19Gag, presumably, at the translational level [Swamynathan S.K. et al. 2000]. (3) YB-1 is contained in a multiprotein complex preventing degradation of the renin mRNA [Skalweit A et al. 2003]. (4) YB-1 selectively binds to the sequence AACCUCUU in the 5'-UTR of the vascular endothelial growth factor mRNA and, along with PTB, another RNA-binding protein, stabilizes this mRNA [Coles L.S et al. 2004]. A similar sequence was found in the 5'-UTR of the interleukin 2 mRNA. Its interactions with YB-1 and nucleolin are necessary for the stabilization of the interleukin 2 mRNA when T cells are activated by JNK [Chen C.Y et al. 2000]. Thus, specific interactions of Y-box-binding proteins with certain mRNA sequences play an important role in the selective regulation of mRNA translation and stability.

1.2.5 PI3K/Akt and YB-1

Several components of the PI3K/Akt signaling pathway are known to be responsible for YB-1 phosphorylation. This pathway is activated by several different mechanisms in cancers, including somatic mutations and amplification of genes encoding key components (Reference).
Akt is often activated in breast cancer but not in normal breast epithelial cells (Kucab et al. 2004) and is able to transform breast epithelial cells into malignant phenotypes (Zhao et al. 2003; Zhang et al. 2003).

Moreover, co-expression of constitutively active Akt1 (CA-Akt1) and mutant polyomavirus T antigen was found to be responsible for mammary tumor formation in nude mice (Hutchinson et al. 2001). Finally, clinical studies have demonstrated a relationship between Akt and tumor relapse and poor prognosis (Bellacosa et al. 1995).

By screening primary tumor tissue microarrays, was found that phosphorylated Akt (pAkt) was associated with YB-1 in breast cancer (Sutherland et al. 2005). Akt1 targets Serine 102 of YB-1, which is located in the CSD (Sutherland et al. 2005). Later on, it was shown that this occur upon IGF-1 stimulation and treatment with Wortmannin, a PI3K inhibitor, fully blocked S102 phosphorylation of YB-1 (Evdokimova et al. 2006). Phosphorylation of YB-1 by Akt was shown to affect YB-1 nuclear trafficking, DNA binding and/or YB-1 mRNA translation. Inhibition of YB-1(S102) phosphorylation, by site directed mutagenesis, impairs YB-1 nuclear translocation (Sutherland et al. 2005).

Studies from Bader and co-workers, in chicken embryo fibroblasts, claim that YB-1 suppresses Akt-mediated oncogenic transformation by inhibiting protein synthesis (Bader et al. 2003; Bader et al. 2005). In striking contrast with the majority of studies on AKT, these authors indicate a role for YB-1 as a tumor suppressor rather than an oncogene.

As previously mentioned, YB-1 nuclear translocation is believed to be regulated by the PI3K/Akt pathway, through S102 phosphorylation. YB-1 was shown to have a non canonical NLS as well as a cytoplasmic retention site (CRS) in the C-terminal domain (Bader et al. 2005). Jürchott and co-workers suggested that both the carboxy-terminal region and the CSD are involved in YB-1 nuclear shuttling (Jürchott et al. 2003). It seems reasonable to postulate that S102 phosphorylation activates the NLS and/or inhibits the CRS function. For instance, phosphorylation of S102 may induce YB-1 conformational change, which masks the CRS and/or reveals the NLS; alternatively, it might control the DNA binding activity of YB-1. Remarkably, wild type YB-1 binds to the 2 kb fragment of the EGFR promoter while the YB-1(A102) mutant was unable to do it (Wu et al. 2006). One may argue that disruption of S102 blocked nuclear translocation of YB-1 thus prevented the binding of YB-1 to the EGFR promoter. However upon careful inspection, mutation of S102 to alanine did not completely block nuclear shuttling of YB-1. In fact, there was still approximately 50% of YB-1(A102) still present in the nuclear compartment (Sutherland et al. 2005).

Finally, it was recently demonstrated that Akt-mediated phosphorylation of YB1-S102 prevented YB-1 from binding to

the capped 5' terminus of mRNA thereby promoting the translation of oncogenes such as IGF-1, VEGF and FOS (Evdokimova et al. 2006). The release of YB-1 from cytoplasmic mRNAs can result in YB1 nuclear translocation (Stenina et al. 2001). The proposed model is that YB-1 phosphorylation by Akt allows latent pro-proliferative gene transcripts to enter into the translational machinery thus supporting the idea that YB-1 is actually working as an oncoprotein (Bader et al. 2003; Evdokimova et al. 2006).
Akt is the first kinase to be shown to regulate YB-1. However, Phospho-motif scanner softwares also predicted that the PI3K p85 subunit may be able to phosphorylate Tyr197 in the C-terminal domain of YB-1.
Interestingly, Tyr197 is located within the NLS of YB-1 (residue 183–202), which raises the question of whether or not YB-1 subcellular localization is regulated by phosphorylation of this residue by PI3K (Bader et al. 2005). This could explain why the PI3K inhibitor wortmannin, by blocking both PI3K and Akt, completely abolished YB-1 phosphorylation induced by IGF-1 stimulation, whereas mutation of S102 to alanine was not able to fully prevent YB-1 phosphorylation (Evdokimova et al. 2006). On the other hand, the glycogen synthase kinase 3 (GSK3), which is negatively regulated by Akt (Diehl et al. 1998), can potentially phosphorylate Ser21 located in the N-terminal domain of YB-1 and affect its transcriptional activity. Coles et al. reported that GSK3β phosphorylated the N-terminus of YB-1, enhancing the ability of YB-1 to repress the vascular endothelial growth factor (VEGF) promoter (Coles et al. 2005). This study also implies the involvement of Akt/GSK3β/YB-1 in tumor angiogenesis. In conclusion, YB-1 phosphorylation via PI3K/Akt signaling is expected to exert a profound effect on tumor pathogenesis and/or progression.

1.2.6 How does YB-1 play a role in cancer?

YB-1 has been shown to be associated with many malignancies including colorectal carcinomas (Shibao et al. 1999), prostate cancer (Gimenez- Bonafe et al. 2004), osteosarcoma (Oda et al. 1998), ovarian serous adenocarcinoma (Kamura et al. 1999; Yahata et al. 2002), lung cancer (Shibahara et al. 2001; Gu et al. 2001), synovial sarcoma (Oda et al. 2003) and breast cancer (Bargou et al. 1997; Janz et al. 2002; Rubinstein et al. 2002; Huang et al. 2005; Wu et al. 2006). Much less is known about the role of YB-1 in childhood cancers. The first study of its kind was published in 2007; the authors showed that YB-1 is a feature of pediatric glioblastoma multiforme (GBM) (Faury et al. 2007). This was discovered initially by comparing pediatric and

adult GBM gene expression profiles. In this study, it was determined that YB-1 is more frequently over-expressed in pediatric GBM compared to adult GBM.
Given that expression of YB-1 is deregulated in so many tumor types, it seemed obvious to think that it plays an important role in cancer development.
YB1 transgenic mice develop mammary tumors and their tumor cells exhibit chromosomal instability and a high content of binucleate cells, most of which were tetraploid.
It was speculated that YB-1 promotes breast tumor formation and/or growth by accelerating cell cycle progression and escaping DNA damage check points (Bergmann et al. 2005). Whether or not YB-1 plays a similar causative role in the development of other types of cancer is unknown at this time. Independently of whether YB-1 is instrumental in the development of cancer or not, it is believed to play an important role in mediating the growth of malignant cells.
The growth and survival of several cancer cells are dependent upon YB-1. For example, melanoma, adenocarcinoma, hepatoma, fibrosarcoma and colon cancer cells die when YB-1 is knocked out with antisense RNA (Swamynathan et al. 2002). It was also revealed an association between YB-1 and expression of two epidermal growth factor receptors, EGFR and Her-2. Wild type YB-1, but not YB-1A102, upregulates the expression of these receptors. Since EGFR and Her-2 are two critical breast carcinoma markers, YB-1 can potentially promote breast cancer growth by stimulating the expression of both receptors (Wu et al. 2006). Accordingly, enforced expression of YB-1 in immortalized breast epithelial cells causes enhanced cell growth associated to the induction of EGFR (Berquin et al. 2005). It can therefore be generally stated that YB-1 is essential for the growth of breast cancer cells. (Bargou et al. 1997; Rubinstein et al. 2002; Janz et al. 2002, Wu et al. 2006).
Much of the work on YB-1 in cancer is centered around its role as a transcription factor while less is known about how this factor affects translation.
As a transcription factor, YB-1 binds to the inverted CCAAT element in the Y-box (Didier et al. 1988; Goldsmith et al. 1993; Norman et al. 2001; Jürchott et al. 2003). YB-1 is also able to recognize Y-boxes without the canonical consensus site (Mertens et al. 1997; Higashi et al. 2003; Lasham et al. 2003), and sequences flanking the Y-box have been suggested to contribute to YB-1:DNA interaction (Didier et al. 1988; Norman et al. 2001). YB-1 up-regulates the transcription of CYCLIN A (Jürchott et al. 2003), CYCLIN B1 (Jürchott et al. 2003), TOPOISOMERASE II α (Shibao et al. 1999), and DNA POLYMERASE α (En-Nia et al. 2005), implying that YB-1

enhances cell growth possibly promoting transcription of both cell cycle progression and DNA replication associated genes. Swamynathan et al. show that cells with targeted disruption of one allele of YB-1 displayed defects in the cell cycle progression and reduction of cell growth (Swamynathan et al. 2002). Although it has been suggested that YB-1 interacts with the proliferating cell nuclear antigen (PCNA; Ise et al. 1999) and DNA polymerase δ (Gaudreault et al. 2004), it is unknown whether or not YB-1 is part of the DNA replication holoenzyme. Due to its role in cell growth, YB-1 is implicated in cancer pathogenesis.
YB-1 was also shown to control the expression of phosphatase PTP1B (Fukada et al. 2003), matrix metalloproteinase- 2 (MMP-2) (Mertens et al. 1997; Mertens et al. 2002), matrix metalloproteinase-12 (MMP-12) (Samuel et al. 2005), collagen α 1(I) (Norman et al. 2001), and collagen α 2(I) (Higashi et al. 2003; Dooley et al. 2006), implying its involvement in mechanism controlling cell adhesion, motility and invasion. The transcriptional activity of YB-1 on MMP-2 was demonstrated to be dependent on the interaction with two other transcription factors, namely the activating protein-2 (AP-2) and p53 (Mertens et al. 2002). Furthermore, YB-1 promotes the expression of several drug resistance genes, including the p-glycoprotein encoded by the multi-drug resistance gene (MDR1) (Goldsmith et al. 1993; Stein et al. 2001), multi-drug resistance related protein-1 (MRP 1) (Stein et al. 2001), as well as the major vault protein (MVP) (Stein et al. 2005).
The ability to regulate genes related to metastasis and drug resistance suggests that YB-1 may be a marker for tumor aggressiveness and may predict the response to chemotherapies.

1.2.7 Why is YB-1 over-expressed in cancer

YB-1 maps on chromosome 1p34.1, and genetic alterations encompassing this region were frequently found in lung, colon and breast cancer (Henderson et al. 2005). Upon closer inspection and fine mapping of 1p34 in lung cancer cell lines, it became clear that the most commonly amplified region was at 1p34.2, which includes the myc loci (Kim et al. 2006).
Similarly, gene amplifications were reported in lung cancer at 1p34.3 to 1p34.4 (Henderson et al. 2005) but again this region does not include the YB-1 gene. Furthermore, there was no evidence for gene amplification at 1p34 in breast cancer cell lines known to over-express YB-1 (Shadeo et al. 2005).
Thus, YB-1 overexpression, in breast and other epithelial cancers, does not appear to be due to a gain in copy number.

Alternatively, YB-1 dysregulation could be due to transcriptional activation. To date, very few studies have addressed the transcriptional regulation of the YB-1 promoter. In one report, multiple E-boxes and GF-boxes on the YB-1 gene promoter were bound by c-Myc, which interacts with p73 to regulate YB-1 expression (Uramoto et al. 2002).
Comparison between YB-1 promoter sequences across different species, revealed multiple transcription factors binding sites, such as those for AML-1, REL and Hunchback (Reference).
In the human YB-1 promoter binding sites for MAX, ARNT, and FOS factors were identified using 90% stringency as the cut-off for this analysis. When the stringency was increased to 100% only N-Myc and Snail were predicted to regulate the YB-1 promoter. The three members of the Myc family, C, N and L-Myc, play important roles in the regulation of cell growth as transcriptional activators when dimerized with MAX. Snail belongs to a superfamily of zinc-finger transcriptional repressors that bind to a six base sequence, CAGGTG, known as the E-box (Batlle et al. 2000). It is involved in cell movement during embryogenesis and has been implicated in promoting epithelial mesenchymal transition (EMT). EMT phenotype arises from the suppression of E-cadherin by Snail resulting in the loss of contact between neighbouring cells (Batlle et al. 2000; Cano et al. 2000). Conversely, Snail over-expression can promote recurrence of primary breast tumors in mice and is associated with decreased patient disease-free survival time in all patient groups (Moody et al. 2005).
YB-1 is also regulated post-translationally. Many transcription factors are indeed regulated by Receptor Tyrosine Kinase (RTK) signal transduction.
Does YB-1 works downstream to the RTK signalling to promote tumor growth and/or resistance to therapy? This remains still an open question.

2. Aims of the work

Increasing evidences associate ΔNp63α dysregulation with squamous cell carcinomas (SCCs) (Fukunishi et al., 2010). On the other hand, loss of ΔNp63α expression was found to be involved in tumor progression and metastasis supporting the idea that ΔNp63α plays a relevant role as a suppressor of metastases (Graziano et al., 2011; Adorno et al., 2009). In addition, downregulation of ΔNp63α by Snail transcription factor, was found to reduce cell-cell adhesion and increase the migratory properties of squamous carcinoma cells (Higashikawa et al., 2007).
To shed light on the role of p63 in epithelial cancer, I focused my work on the study of the interaction between ΔNp63α and the YB-1 oncoprotein, a newly identified ΔNp63α partner, that is already known to be an important marker of tumorigenesis in several malignancies (Sutherland et al., 2005).
My work consists of four distinct phases:

I. Study of the interaction between p63 isoforms and YB1 using *in vivo* and *in vitro* approaches. Determination of the mutual effects on the expression levels, stability and sub-cellular localization.

II. Analysis of the effects of p63 and YB-1 on PI3KCA gene transcription and ability of YB-1 to bind specific mRNA into the cytoplasm.

III. Determination of the effect of p63 on the ability of YB-1 to promote cell migration in tumor cells.

IV. Analysis of YB-1 and p63 gene expression in primary cells and regenerating corneal tissue.

The final aim of this work is to support relevant knowledge to develop new pharmacological agents and therapies that impinging on YB-1 and/or ΔNp63α changes during cancer progression can help to fight tumor progression.

3. RESULTS

3.1 Analysis of Y-Box Binding Protein 1 expression in different cancer cell lines.

In the laboratory where I've carried out my PhD thesis, a comprehensive screening for ΔNp63α interactors was performed using functional proteomics. Through this approach a total of 53 potential ΔNp63α binding partners were identified. Proteins with DNA/RNA binding activity were highly represented and the Y-Box binding protein 1 (YB-1 or YBX-1) was one of them (Amoresano et al., 2010). Figure 3 shows a graphic representation of p63 potential interactors grouped on the basis of their function.

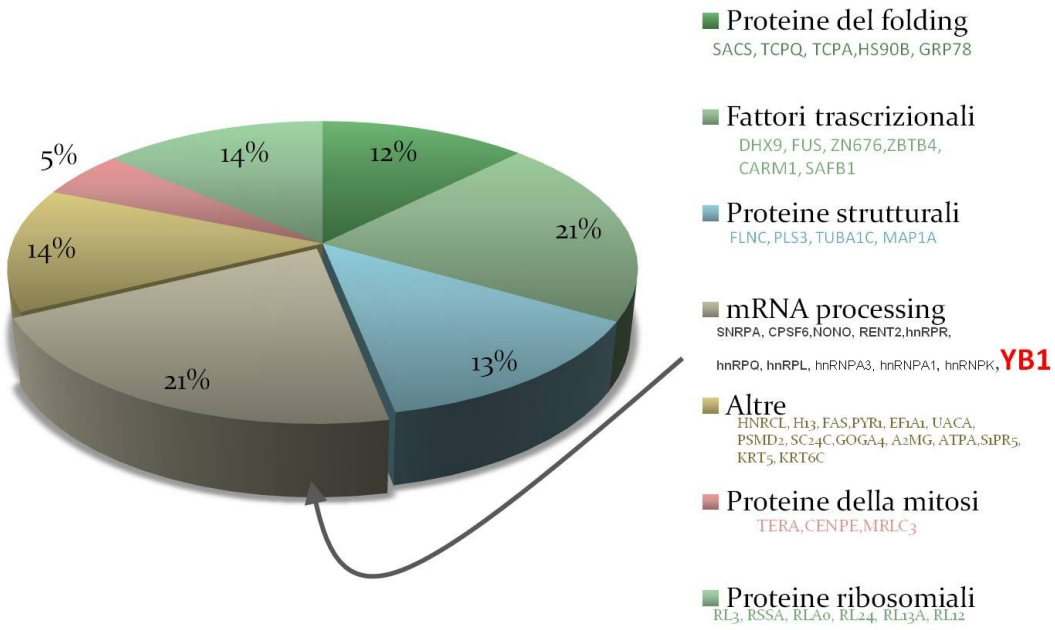


Figura 3 The p63 interactors . Schematic representation of p63 interactors isolated by co-immunoprecipitation and subsequent analysis by mass spectrometry

I first analyzed the expression profile of YB1 in a set of different cancer cell lines. YB-1 was abundantly expressed in all cell lines tested. Immunoblot analysis of total cell extracts using antibodies against the N-terminus of YB-1 showed a series of immunoreactive bands with an apparent molecular weight of 50 (YB-1-50), 43 (YB-1-43) and 36 KDa (YB-1-36) (Figure).
As previously reported, different proteolytic forms of YB-1 show a different subcellular localization (reference). Therefore, using a protocol for nuclear/cytoplasmic cell extract fractionation,

based on the use of a linear gradient of sucrose, I separated the nuclear and cytoplasmic fractions of YB-1. Immunoblot analysis was then performed using antibodies against the N-terminal region of YB1. Depending on the cell line, I found considerable differences in the electrophoretic pattern of YB-1 immunoreactive bands not only respect to their relative abundance but also to their distribution between the nuclear and cytoplasmic compartments.

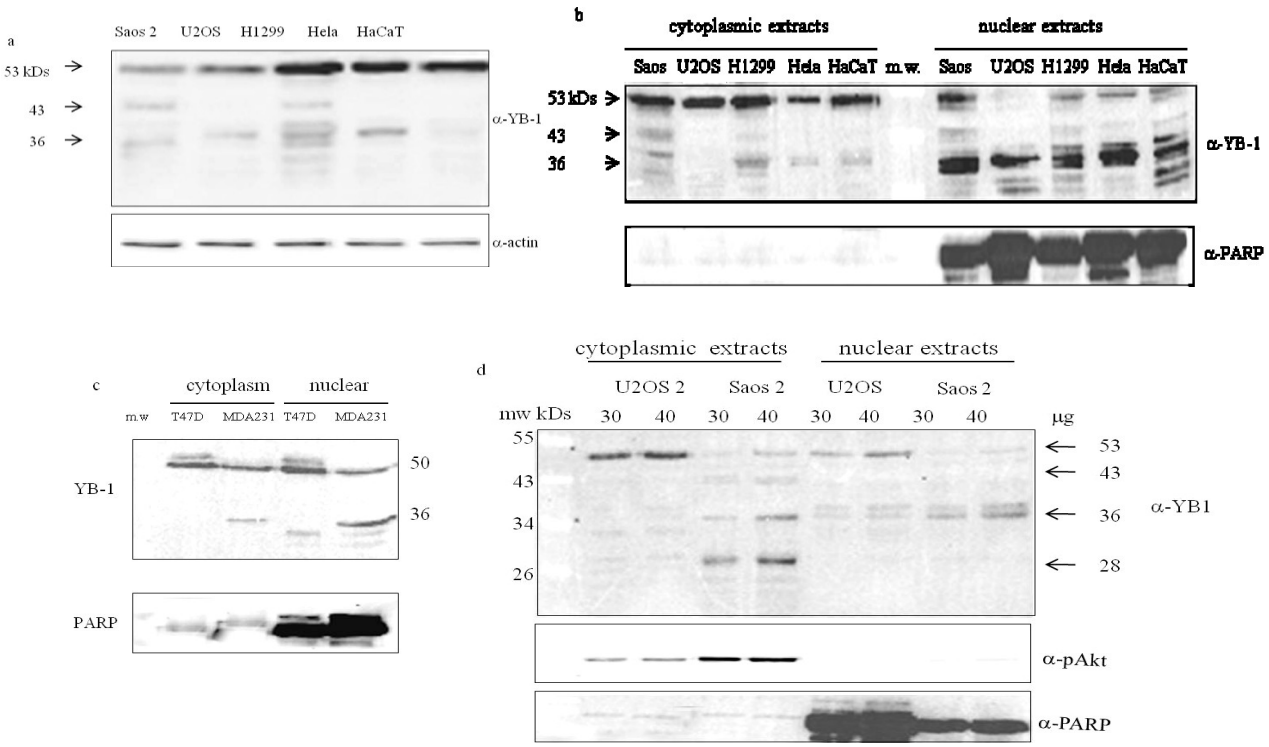


Figura 4 (a) Total cell extracts(20 μg) from the indicated cell lines were subjected to Immunoblot analysis using antibodies against the N-terminal region of YB-1. Actin was used as loading control **(b;c)** Nuclear or cytoplasmic extracts (20μg) from the indicated cell lines were separated by SDS PAGE and subjected to immunoblot. Proteins were detected with antibodies against the N-terminal region of YB-1, as indicated. **(d)** Nuclear or cytoplasmic extracts (30 to 40 μg)from U2OS and Saos2 cells were separated by SDS PAGE and subjected to immunoblot using a polyclonal antibody against the full length YB-1 protein. PARP and pAKT were used as nuclear and cytoplasmic control respectively to check for cross-contamination. Images were acquired with CHEMIDOC (BIORAD) and analyzed with the Quantity-ONE software.

By treating cells with the proteasome inhibitor MG132, the YB-1-43 and YB-1-36 bands disappeared while YB-1-50 accumulated, thus suggesting that the smaller forms of YB-1 were due to specific proteolityc cut (Figure 5). The experiment also shows that p53 enforced expression does not significantly changes the YB-1 electrophoretic pattern in this cell context

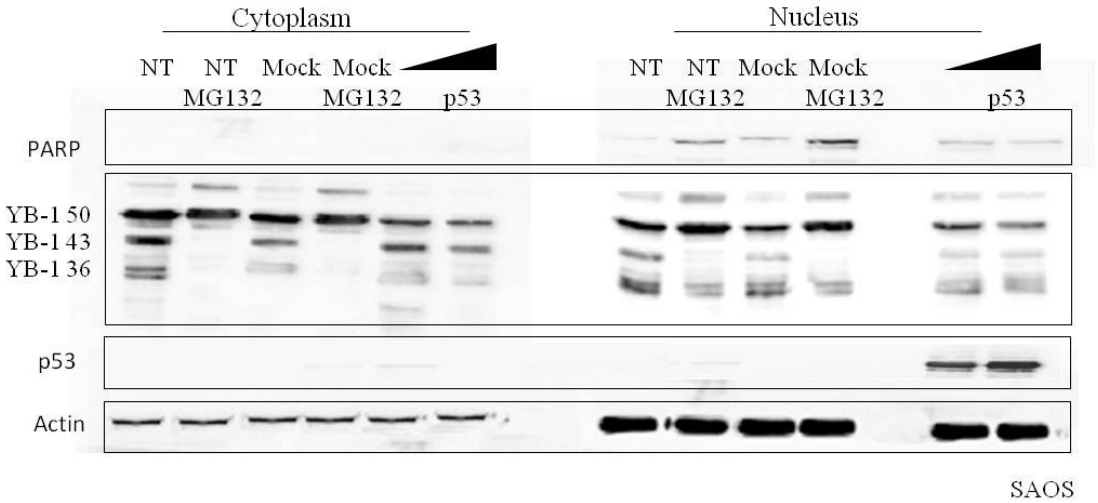


Figura 5 YB-1 immunoreactive protein bands are the results of specific proteolityc cuts. Untreated, mock or p53-transfected (5-8 μg of p53 expression plasmid) Saos2 cells were grown in cell culture medium supplemented or not with MG132. 24h after transfection, cell extracts were fractionated to obtain cytoplasmic and nuclear fractions. Nuclear and cytoplasmic extracts (20μg) were separated by SDS PAGE and subjected to immunoblot. Proteins were detected with anti-YB-1 antibody. Parp was used as nuclear control to check for cross contamination. Actin was used as loading control.

3.2 YB-1 interacts with ΔNp63α *in vitro* and *in vivo*.

To confirm the interaction between p63 and YB-1 have performed reciprocal co-immunoprecipitation experiments in HaCaT cell, expressing high levels of endogenous ΔNp63α and p63 transfected H1299 cells that normally lack both p63 and p53. As shown in Figure 6, YB-1-50 was efficiently immunoprecipitated with p63 specific antibodies in both cellular contexts. However, as we could not observe ΔNp63α/YB-1 immunocomplexes performing the reciprocal experiment (Figure 6b), we transfected HaCaT cells with a plasmid encoding the Flag epitope fused to YB-1. As shown in Figure 6c ΔNp63α was immunoprecipitated with anti-Flag antibodies, only in cells expressing Flag-YB1. To determine if the interaction can occur with other p63 isoforms we transfected H1299 cells with the p63α or γ isoforms both in the TA or ΔN version to perform co-immunoprecipitation experiments. Remarkably, I found YB-1 present in immunocomplexes with α isoforms (left panel) but not γ (right panel). So we argued that YB-1 interacts with the p63 α-specific SAM or post-SAM domain.

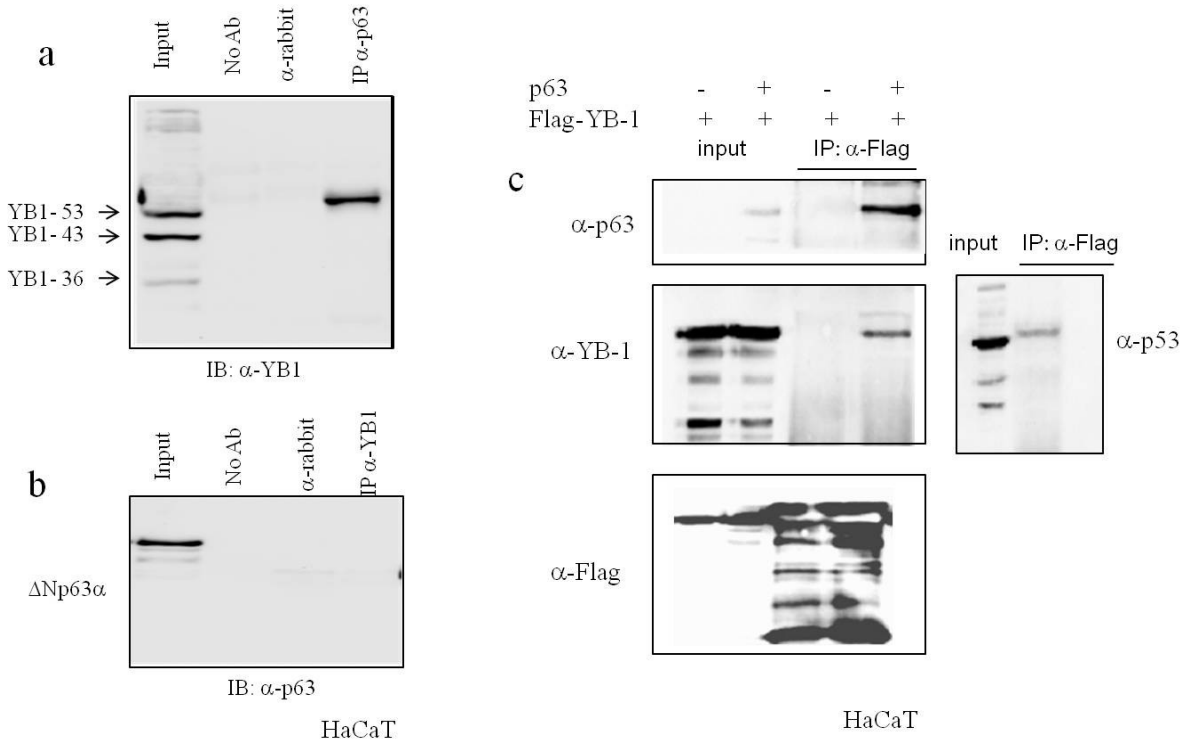


Figura 6 ΔNp63α interacts with YB-1. **(a)** Extracts from HaCaT cells were immunoprecipitated with anti-p63 **(a)** or anti-YB-1 antibodies **(b)** and the immunocomplexes were blotted and probed with anti-YB1 or anti-p63 antibodies, as indicated. A sample with no antibody (no Ab) or irrelevant α-mouse antibodies were included as controls. **(c)** Extracts from untransfected and 3XFlag-YB-1 (0.5 μg) transfected HaCaT cells were immunoprecipitated with anti-Flag antibodies. The immunocomplexes were blotted and probed with anti-p63 (upper panel) or anti-YB-1 antibodies (lower panel). Anti-p53 was included as positive control of interaction (right pannel)

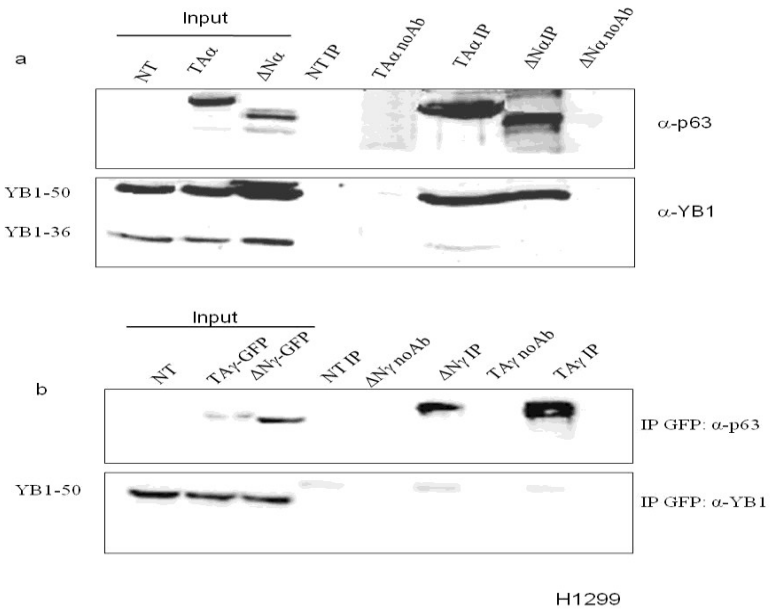


Figura 7 YB1 interacts with α but not with γ isoforms of p63. (a) H1299 cells were transiently transfected with ΔNp63α or TAp63α plasmid (5 µg) and GFP-tagged ΔNp63γ or TAp63γ expression vectors. At 24 hrs, equal amount (1 mg) of extracts were immunoprecipitated with anti-p63 or anti-GFP antibodies. The immunocomplexes were blotted and probed with anti-p63 and anti-YB-1 antibodies. A sample with no antibody (no Ab) were included as negative

The co-immunoprecipitation assay by itself does not allow to determine whether the association between two proteins is direct or mediated by other proteins. To address this point, I expressed YB-1, ΔNp63α and ΔNp63γ recombinant proteins in bacteria. After the purification procedure, the proteins were subjected to Far Western, an *in vitro* interaction assay.

ΔNp63α and γ cDNAs from the pcDNA3myc plasmid backbone were subcloned into the pRSETA bacterial vector under the control of T7 promoter. This plasmid also encodes a sequence of polihistidine (His-tag) for protein purification.

E. Coli DH5α strain was transformed with pRSETA-ΔNp63γ, pRSETA-ΔNp63α and pRSETA-YB1 for plasmid amplification. Finally, the purified p63 and YB-1 encoding plasmids were transferred to E. coli BL21 (DE3) strain for IPTG-inducible protein expression. Bacterial extracts were then analysed for recombinant p63 or YB-1 protein expression. Upon induction with 1mM IPTG, ΔNp63α and γ proteins were present in the supernatant of cells (Figure 8) while YB-1 formed inclusion bodies. Therefore, an additional His-tag affinity chromatography step was necessary to purify YB-1. The quality of the purified proteins was checked by reciprocal western blot experiments using anti-hys, anti-YB1 or anti-p63 antibodies.

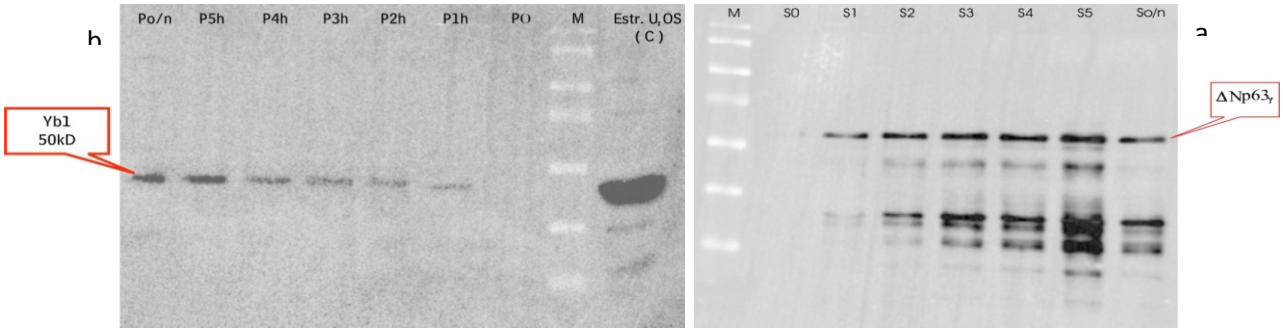


Figura 8 Analysis of expression of ΔNp63γ and YB-1 recombinant proteins: BL21 AI cells of *E. coli* strain transformed with vectors pRSETA ΔNp63γ (a) or YB-1 (b) were grown in the LB medium at 0.2 O.D. and then induced with 1mM IPTG. 1mL of cell growth was lysed and 10µg of supernatant (a) or pellet (b) for each point were analyzed by SDS-PAGE followed by Western blot analysis with anti-p63 or anti-YB-1 antibodies. 20µg of U2OS cell extract were included as control.

Then I performed a Far-Western blotting using ΔNp63α, ΔNp63γ or YB-1 recombinant proteins. Increasing amounts of proteins were subjected to SDS-PAGE and transferred to PVDF membranes. Proteins were quickly denatured using 6M urea and re-natured gradually allowing them to regain their native conformations. The filters were then incubated with appropriate amounts of the potential protein-partner which was used as a probe. After extensive washing, the filters were subjected to immunoblot with antibodies directed against the protein-probe. As shown in Figure X a, b, I found that YB-1 interacts with p63 when ΔNp63α, but not ΔNp63γ, was used as a probe. I confirmed this result by performing the reciprocal experiment (Figure 9)

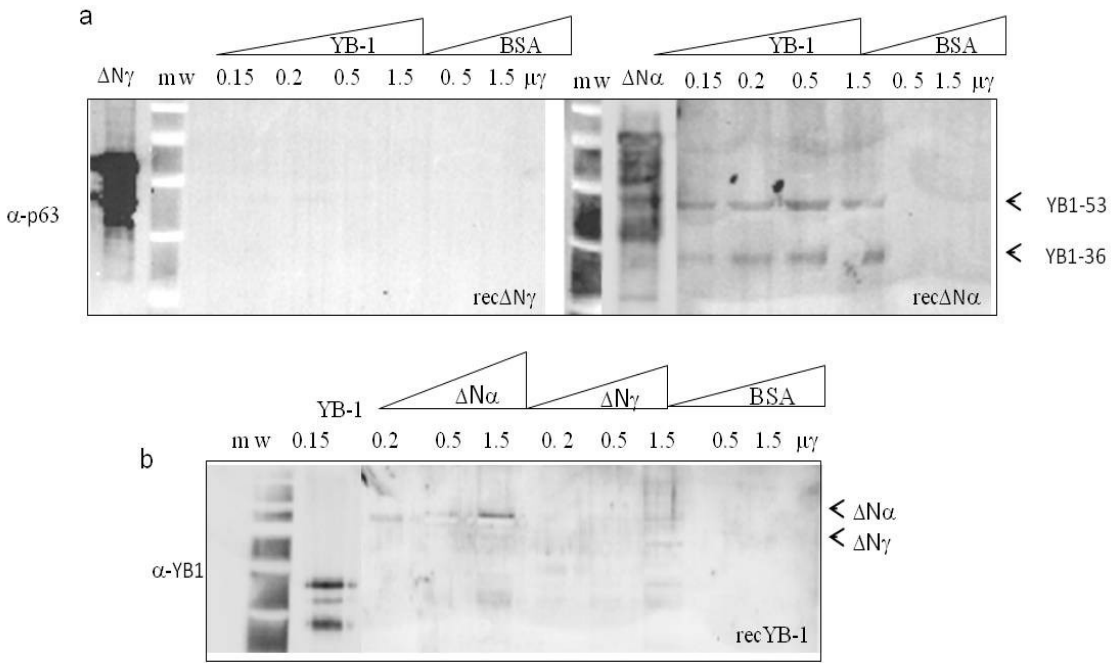


Figura 3 Far-western analysis. (a) The indicated amount of purified recombinant YB-1, and BSA were subjected to SDS-PAGE, transferred to PVDF membrane and probed with 0.8 μg/ml of recombinant ΔNp63γ (left panel) or ΔNp63α (right panel). The membrane was washed and subjected to immunoblotting towards p63 antibodies followed by ECL. Recombinant ΔNp63α or ΔNp63γ (100 ng) were used as positive controls. Correct loading of the proteins was monitored by coomassie staining; m.w. (molecular weight markers). **(b)** The indicated amounts of purified recombinant ΔNp63α, ΔNp63γ, YB-1 and BSA were subjected to SDS–PAGE and transferred to PVDF membrane. The membranes were incubated with purified recombinant YB-1. The filter was incubated with anti-YB1 antibodies, washed and subjected to immunoblotting towards YB-1 followed by ECL detection.

3.3 p63 and YB-1 functional association: do they exert a reciprocal control on their expression levels?

A physical association between two proteins can potentially influence their expression levels by several distinct mechanisms (review reference). I investigated whether p63 enforced expression was able to modulate YB-1 protein levels and vice-versa. H1299 cells were transfected with increasing amounts of vectors encoding TAp63α,TAp63γ, ΔNp63α or ΔNp63γ. After 48 hours, cell extracts were prepared and analysed by immunoblot. As shown in Figure 10, p63 over-expression did not significantly alter YB-1 expression levels. I obtained similar results in different cancer cell lines.

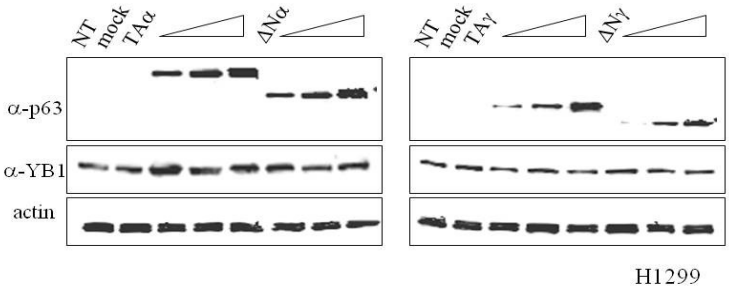


Figura 10 The p63 enforced expression does not induce significant changes of YB-1 expression levels: H1299 cells were transfected with increased amounts (0.5, 1 and 1.5 μg) of α or γ p63 expression plasmids or pcDNA3 control vector (mock). Cell extracts were prepared and analysed by Western blot using anti-p63 or anti-YB-1 antibodies. No substantial effects of p63 on the expression level of endogenous YB-1 were detectable. Actin was used as a loading control.

An alternative approach to determine whether p63 exerts a control on YB-1 expression level was to knock-down p63 expression in HaCaT and Fadu cells, expressing high levels of endogenous p63. As shown in (Figure 11, a) YB-1 was only slightly reduced in cells transfected with p63 siRNA. Using a similar approach, I did not detect significant changes of ΔNp63α expression level in HaCaT cells partially depleted of YB-1 by siRNA. (Figure 11, b)

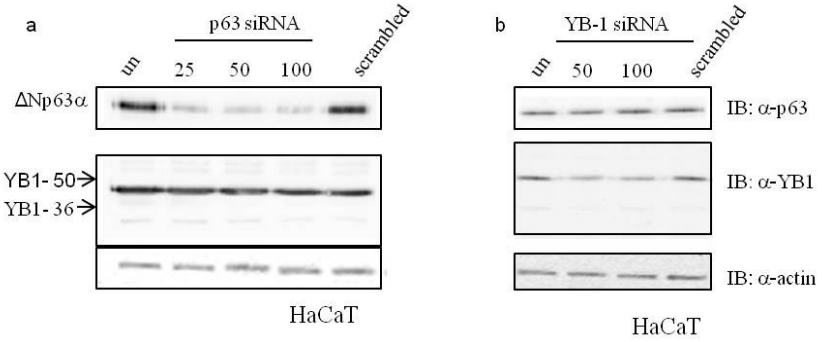


Figura 11 Mutual effects of specific silencing: Hacat cells were treated with 25, 50 and 100nM p63 **(a)** or YB-1 **(b)** specific siRNA for 48h. 100nM siLUC were used as negative control (scrambled). 20μg of cells lysates were separated by SDS PAGE and subjected to immunoblot. Proteins were detected with specific antibodies, as indicated. Actin was used as loading control

3.4 ΔNp63α induces YB-1 nuclear accumulation

YB-1 shuttles between the nucleus and cytoplasm exerting different and sometimes conflicting role in either cellular compartments (Ken Matsumoto, Alan P Wolffe 1998). In normal cells, however cytoplasmic YB-1 is largely prevalent. On the other hand, p63 is a transcription factor that localizes predominantly into the nucleus. However, in particular conditions, p63 immunoreactive stain was detected into the cytoplasm of breast epithelial cells (Bratthauer GL 2005).
We wanted to address whether or not p63 was able to influence YB-1 subcellular localization and colocalize with YB-1 in the same subcellular compartment.
To this aim, I performed immuno-localization assays in H1299 cells. To avoid background problems, I decided to use direct fluorescence on YB-1-GFP transfected cells as YB-1 is highly and constitutively expressed in all mammalian cells.
As shown in Figure 12., the large majority of cells transfected with YB-1-GFP showed a strong fluorescence signal in the cytoplasm (95%) while in the remaining (5%) the signal was distributed between the nucleus and cytoplasm (Figure 3b). Interestingly, ΔNp63α transfected cells showed a significant increase in the number of cells showing YB-1GFP exclusively nuclear or equally distributed between the nucleus and cytoplasm.
In particular, more than 40% of cells showed intense nuclear GFP signal (Figure 13a upper panel and 13b left panel), an additional 20% exhibited YB-1-GFP almost equally distributed between the nucleus and cytoplasm while the remaining 40% exhibited cytoplasmic GFP-YB1 (Figure13c). Conversely, YB-1-GFP subcellular distribution was almost unaffected by ΔNp63γ expression (Figure13a lower panel and 13b right panel). The graph in the figure was obtained by counting 10^2 cells for each experimental point and classifying them on the basis of YB1-GFP localization: cytoplasmic, nuclear-cytoplasmic, exclusively nuclear. Similar data were obtained using the YB-1 Flagged protein meaning that the GFP epitope does not alter YB-1 cellular distribution.

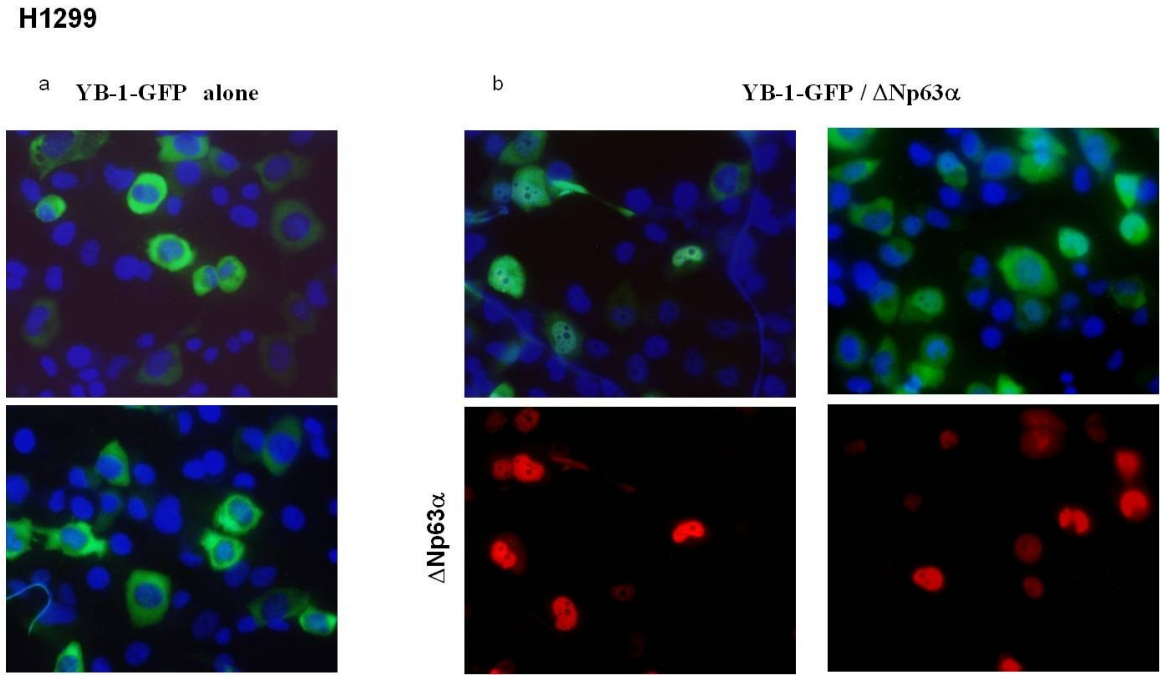


Figura 4 ΔNp63α induces YB-1 nuclear accumulation: H1299 cells were seeded in at 60% confluency (2.3 x 105) on 24 x 24 mm sterile coverglasses placed in 60 mm dishes and transiently transfected with GFP-YB1 expression vector (0.3 µg) alone **(a)** or with 1 µg of ΔNp63α expression vector **(b).** Cells were fixed and subjected to indirect immunofluorescence as indicated in Materials and Methods. GFP-YB1 was detected by direct fluorescence and p63 by using anti-p63 antibodies and secondary anti-mouse Cγ3-conjugated (red). 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei (*blue*). YB-1 GFP/DAPI merged image were generated with Adobe Photoshop CS3 software.

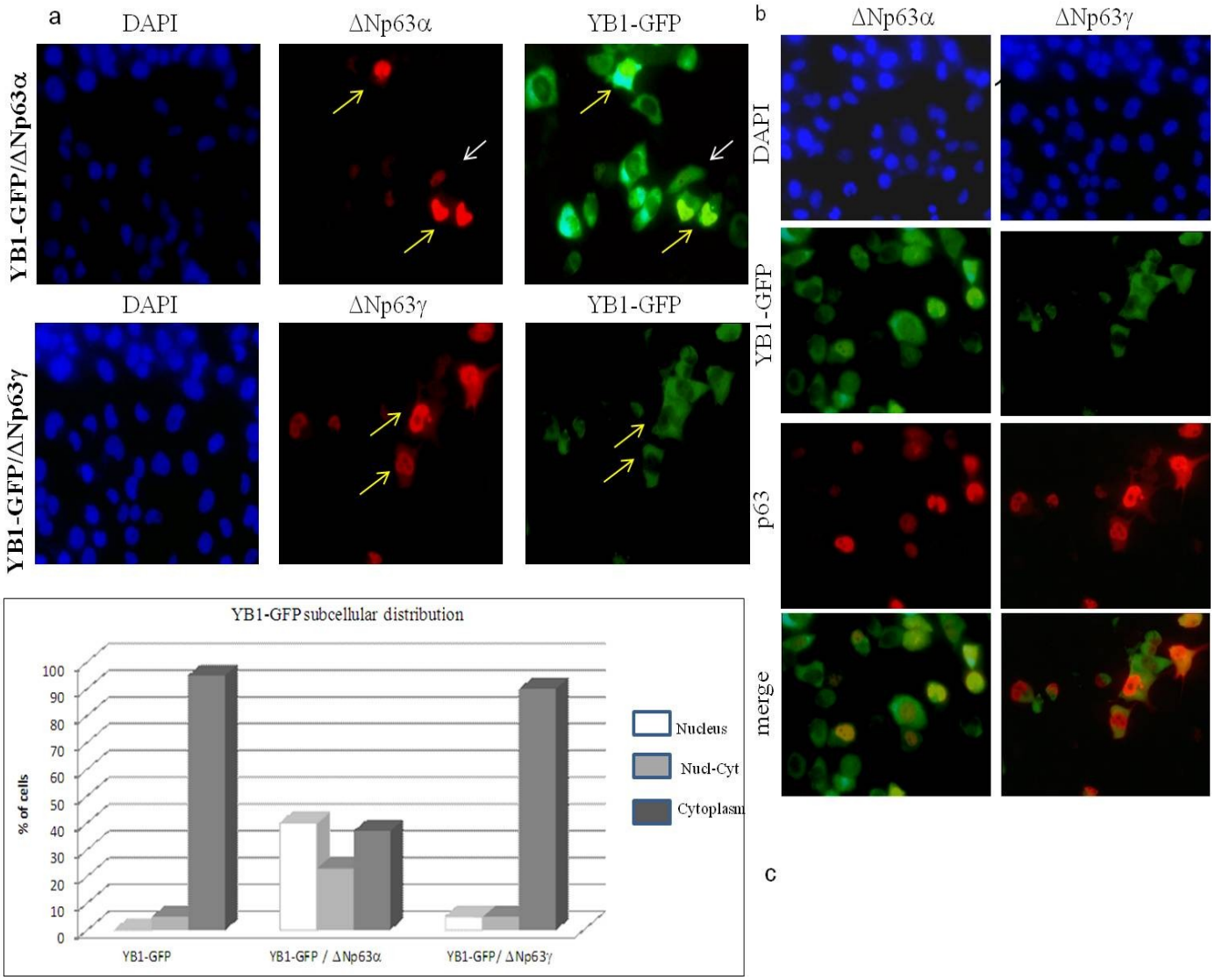


Figura 13 ΔNp63α induces YB-1 nuclear accumulation. (a and b) H1299 cells were seeded in at 60% confluency (2.3 x 105) on 24 x 24 mm sterile coverglasses placed in 60 mm dishes and transiently transfected with GFP-YB1 (0.3 µg) and 1 µg of ΔNp63α or ΔNp63γ expression vectors. Cells were fixed and subjected to indirect immunofluorescence as indicated in Materials and Methods, GFP-YB1 was detected by direct while p63 was detected using anti-p63 antibodies and secondary anti-mouse Cy3-conjugated (red). Merge between p63 and GFP-YB1 are indicated by yellow arrows. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei. Both ΔNp63α and ΔNp63γ exhibit nuclear localization. But only ΔNp63α determines YB-1 nuclear accumulation . **(c)** Plot showing the percentage of nuclear (white plot), nucleo-cytoplasmic (grey) and cytoplasmic (dark grey) GFP-YB-1. Each experimental point is the average of counts performed on 100 cells in 5 independent fields.

Given the importance of YB-1 and p63 deregulation in breast cancer progression (Huang et al., 2005), I queried if ΔNp63α was able to cause YB-1 nuclear translocation and accumulation in breast cancer cells. MDA-MB-231 is a cell line derived from aggressive, basal-like breast carcinoma (BLBC) expressing high level of endogenous YB-1, but no detectable p63. I transiently transfected ΔNp63α in these cell lines and detected

endogenous YB1 by indirect-immunofluorescence. As shown in Figure 14, intense staining of endogenous YB-1 was present in the nucleus of more than 90% of cells expressing ΔNp63α (Figure 14, yellow arrows).

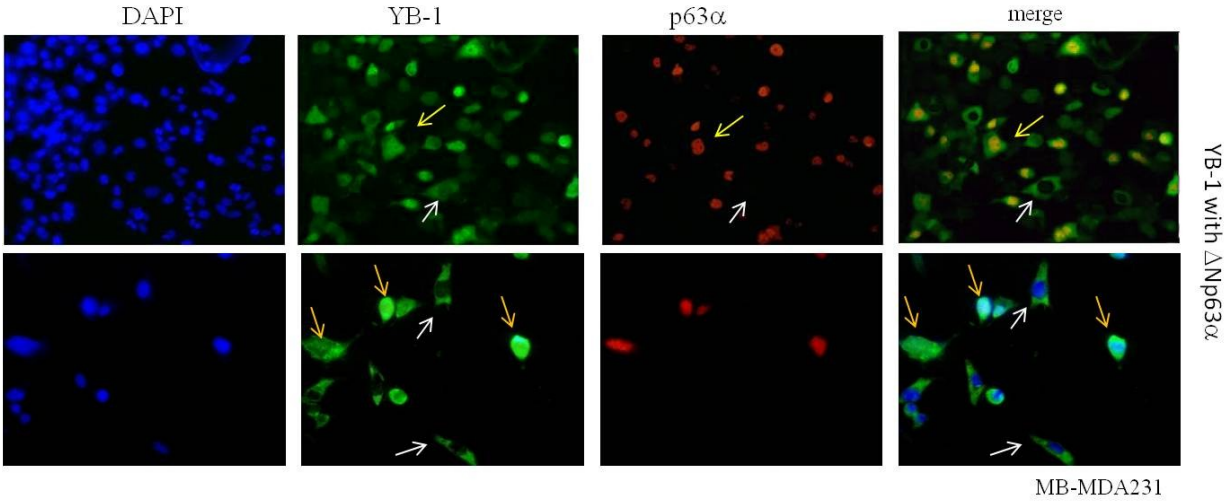
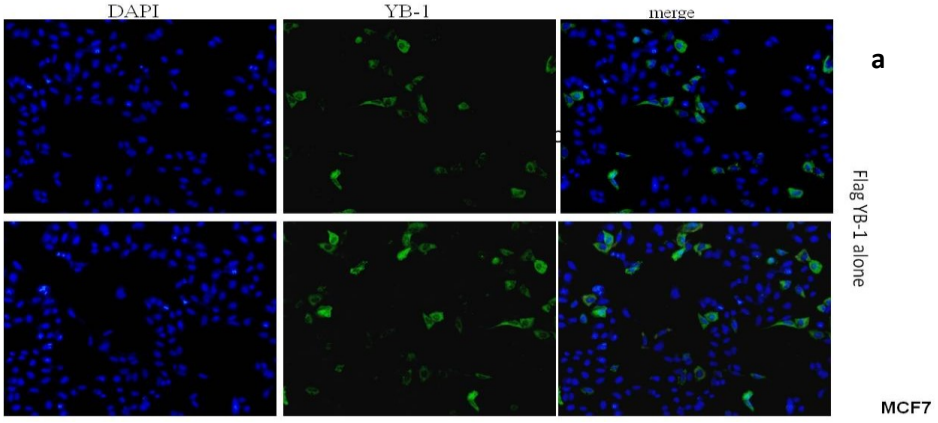


Figura 14 ΔNp63α induces nuclear accumulation of endogenous YB-1 in breast cancer cells. MDA-MB-231 cells were seeded at 60% confluency (2.3 x 105) on 24 x 24 mm sterile cover-glasses placed in 60 mm dishes and transiently transfected with 1 µg of ΔNp63α expression vector. Cells were fixed and subjected to double indirect immunofluorescence using rabbit primary YB-1 antibody and Fitch-conjugated secondary antibodies (green). P63 protein was detected using mouse anti-p63 and Cy3-conjugated secondary antibodies (red). 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei (blue). More than 90% of cells expressing ΔNp63α showed nuclear YB-1 (yellow arrows). Cytoplasmic YB-1 was detected in cells bearing low or no ΔNp63α expression (white arrows).

Then, I transfected FLAG-YB-1 construct alone or with ΔNp63α plasmid in MCF7 cells, derived from non aggressive basal-like breast carcinoma (BLBC). In MCF7 cells, FLAG-YB-1 was prevalently located in the cytoplasm. However, similar to what observed in MDA-MB-231 cells, FLAG-YB-1 was nuclear in almost all cells expressing detectable levels of ΔNp63α (Figure 15).



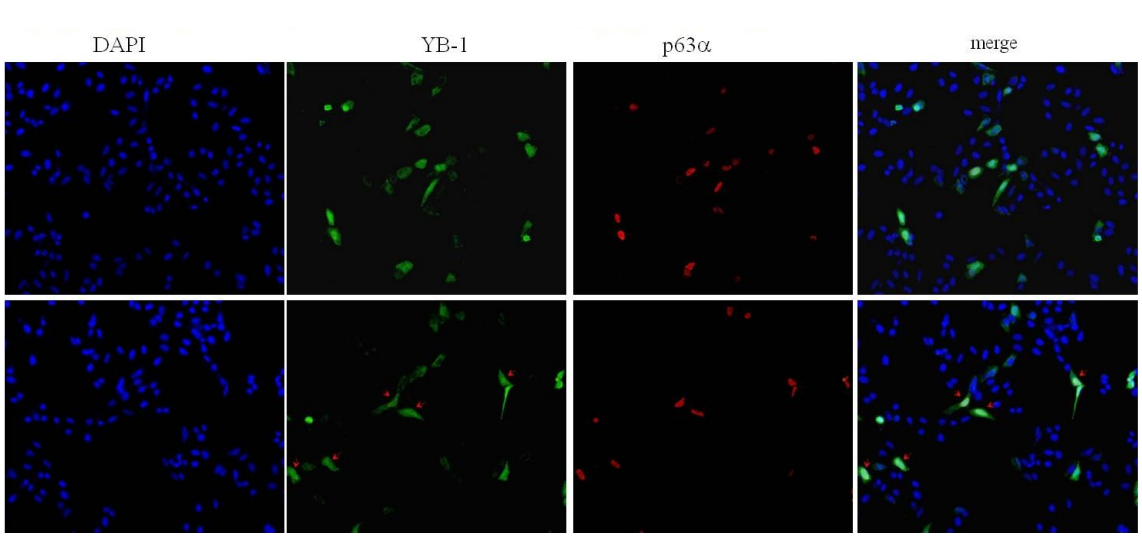


Figura 5 ΔNp63α induces nuclear accumulation of Flag YB-1 in breast cancer cells. (a) MCF7 cells were seeded at 60% confluency (2.3 x 10^5) on 24 x 24 mm sterile coverglasses placed in 60 mm dishes and transiently transfected with 0,3 µg of FLAG YB-1 expression vector. Cells were fixed and subjected to double indirect immunofluorescence using anti-Flag antibody and secondary anti-rabbit Fitch-conjugated (green). The FLAG YB-1, as GFP YB-1 and endogenous YB-1, exhibits a predominantly cytoplasmic localization. **(b)** MCF7 cells (2.3 x 10^5) were seeded as described in **(a)** and transiently transfected with Flag YB1 (0,3 µg) and a fixed amount of pcDNA3/ΔNp63α expression vectors (1µg). Cells were fixed and subjected to double indirect immunofluorescence using anti-Flag antibody and secondary anti-rabbit Fitch-conjugated (green) P63 protein was detected using mouse anti-p63 and Cy3-conjugated secondary antibodies (red). 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei (blue). More than 90% of cells expressing ΔNp63α showed nuclear YB-1 (red arrows).

To confirm the observed effect of ΔNp63α on YB-1 nuclear accumulation, using a different experimental approach, I performed nuclear/cytoplasmic fractionation of extracts from MDA-MB-231 cells transfected with ΔNp63α or ΔNp63γ. By immunoblot with antibodies against the YB-1 N-terminal region, YB-1 was detectable as 50 and 36 kDa immunoreactive bands. YB-1/50 kDa was present both in nuclear and cytoplasmic fractions while YB-1/36 kDa was detectable only in the nuclear fraction (Figure 16a). However, in cells transfected with ΔNp63α, but not ΔNp63γ, we observed an increase of nuclear YB-1 (Figure 16a).
Similar results were obtained in U2OS cells, expressing wild type p53, (Figure 16d) and in a p53-null context such as in Saos2 and H1299 cells, where co-transfection of ΔNp63α caused the nuclear accumulation of endogenous YB-1 and transfected Flag-YB-1 (Figure 16 b and c).
Interestingly, I repeatedly observed an increase of the signal corresponding to phosphoserine at position 473 of Akt1 in the cytoplasm of ΔNp63α, but not ΔNp63γ, transfected cells suggesting that ΔNp63α was, in some way, involved in the activation of PI3K/Akt signalling (Figure 16 a and b).

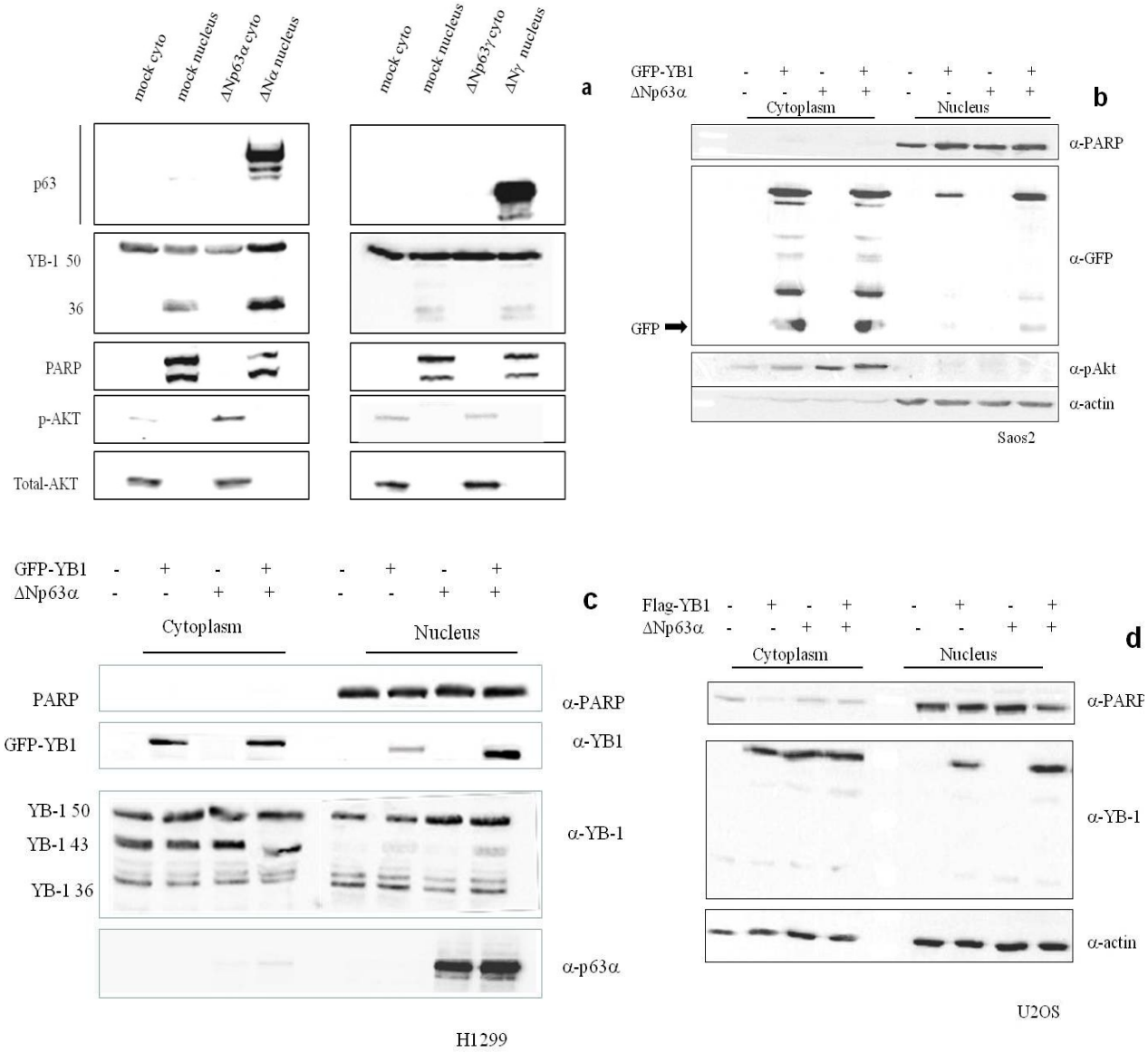


Figura 16 ΔNp63α, but not ΔNp63γ promotes YB-1 nuclear accumulation. MDA-MB-231 cells were transiently transfected with a fixed amount (5 µg) of an empty vector (mock), ΔNp63α or ΔNp63γ expression vector in 100 mm dishes. 24h after transfection, cell lysates were fractionated to obtain cytoplasmic and nuclear fractions. 20µg of nuclear and cytoplasmic extracts were separated by SDS PAGE and subjected to immunoblot. Proteins were detected with specific antibodies, as indicated. PARP and total AKT were used as nuclear and cytoplasmic control respectively, to check for cross-contamination. Images were acquired with CHEMIDOC (BIORAD) and analyzed with the Quantity-ONE software. SAOS2 cells**(b)** and H1299 cells**(c)** were transfected with an empty vector and a fixed amount of pEGFP/YB-1 encoding plasmid alone or with ΔNp63α plasmid. After subcellular fractionation, 20 µg of protein extract were subjected to immunoblot with the indicated antibodies. **(d)** U2OS cells were transfected with an empty vector and a fixed amount of pFlag/YB-1 encoding plasmid alone or with ΔNp63α plasmid. After subcellular fractionation, 20 µg of protein extract were subjected to immunoblot with the indicated antibodies. PARP and pAKT were used as nuclear and cytoplasmic control respectively was used as nuclear control, to check for cross-contamination. Actin was used as loading control.

3.5 Role of Akt in ΔNp63α-mediated YB-1 nuclear translocation

Akt/PKB is a serine/threonine kinase that promotes tumor cell growth by phosphorylating transcription factors and cell cycle proteins. YB-1 is a phospho-substrate for Akt. The major site phosphorylated by Akt on YB-1 was mapped to Serine 102 and disruption of this residue was correlated with inhibition of YB-1 nuclear translocation (Sutherland et al., 2005; Evdokimova et al., 2006). Because of the ability of ΔNp63α to trigger Akt phosphorylation, we looked at the effect of Akt activation in YB-1 nuclear accumulation. We co-transfected YB-1GFP with activated Akt (CAAkt) (Rao et al., 2005). As shown in Figure 8a, upon intense Akt activation, the YB-1GFP fluorescence signal appeared to be reduced and confined to the perinuclear compartment. However, when CA-Akt was cotransfected with ΔNp63α, the whole number of cells showing intense GFP-YB1 nuclear signal also expressed ΔNp63α while cells showing perinuclear GFP-YB1 expressed a barely or not detectable level of ΔNp63α (Figure 17).

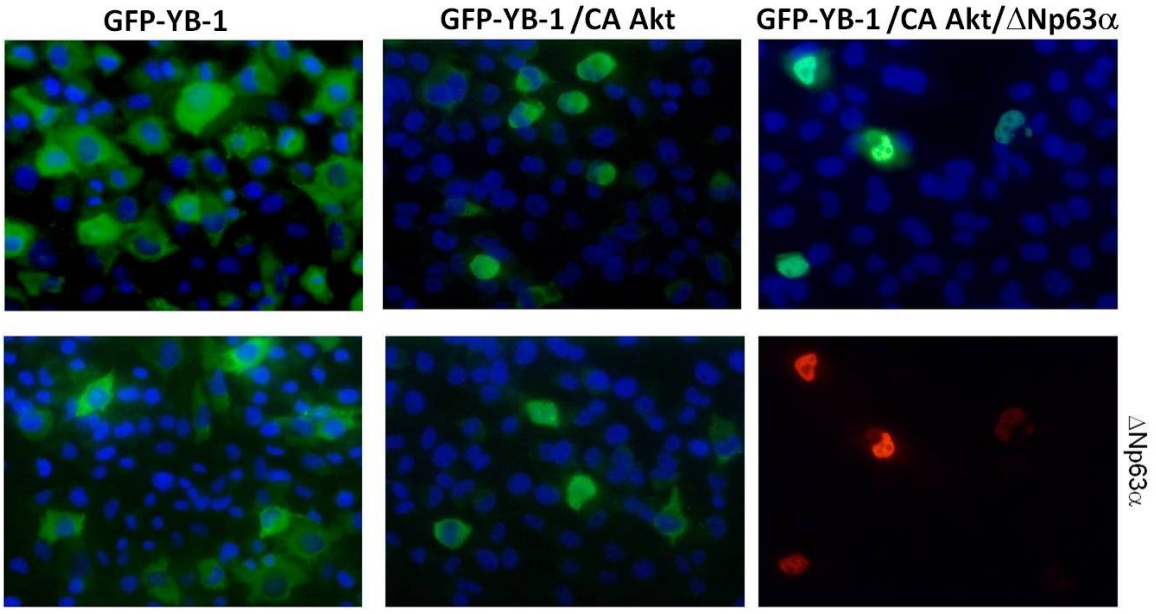


Figura 17 Akt activation does not promote, by itself, YB1 nuclear accumulation. H1299 cells, seeded at 60% confluency (2.3 x 105) on a 24 x 24mm steryl coverglass in 60mm dishes, were transiently transfected with GFPYB-1 (0,3 μg) plasmid alone or with CA-Akt1 (1 μg) with or without ΔNp63α plasmid (1 μg), as indicate in figure, Cells were fixed and subjected to direct immunofluorescence. P63 protein was detected using mouse anti-p63 and Cy3-conjugated secondary antibodies (red) 4′,6-Diamidino-2 phenylindole (DAPI) was used to stain nuclei (blue). Merge was generated by Adobe Photoshop CS3 software.

Like the wild type, the phosphorylation-defective FlagYB-1S102A did not translocate to the nucleus when cotransfected with CA-AKT. However, it was equally able to accumulate into the nucleus in cells expressing ΔNp63α (Figure 18) suggesting that phosphorylation of this residue was not required for ΔNp63α-mediated YB-1 nuclear accumulation.

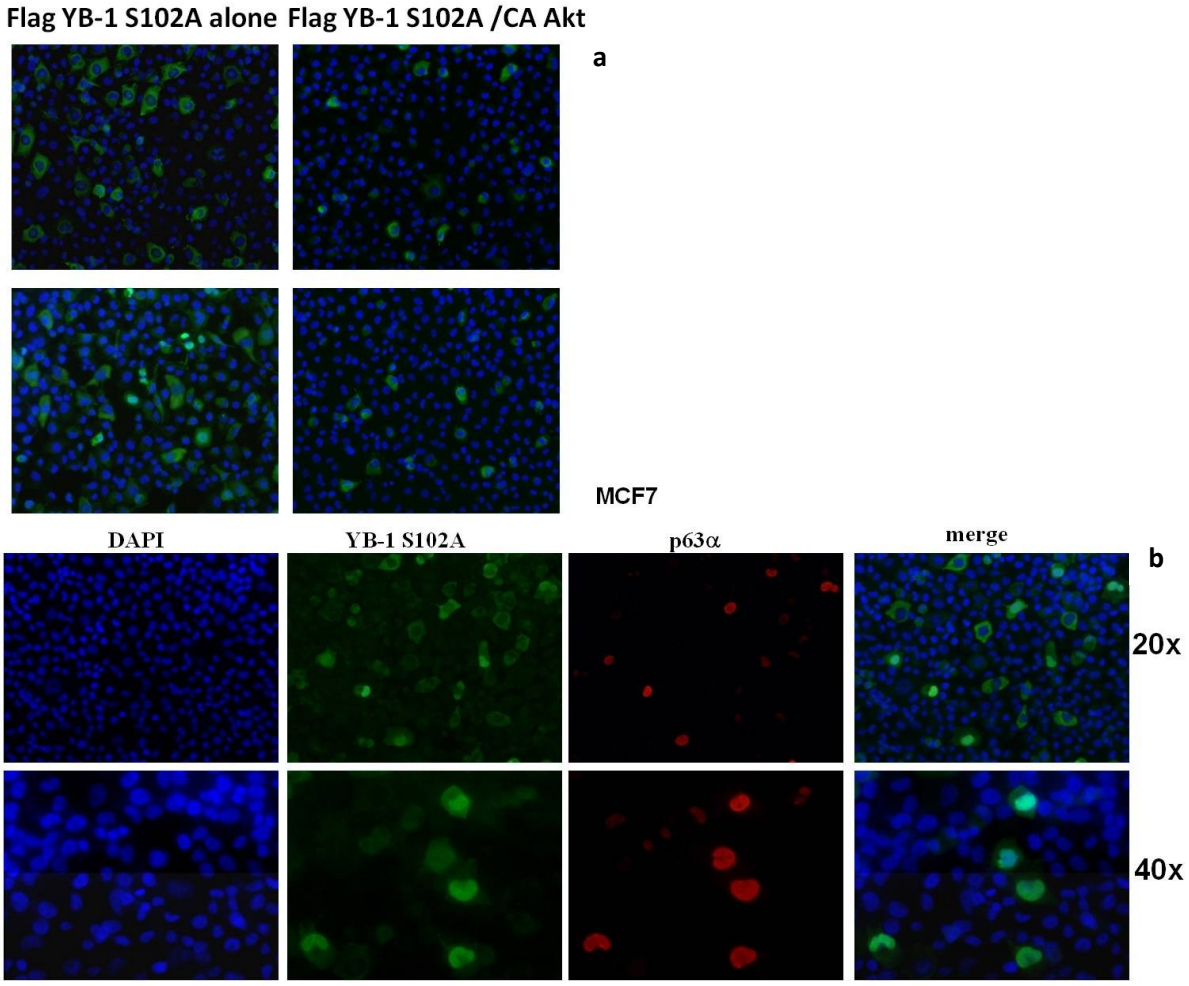


Figura 18 Akt activation does not promote, by itself, YB1S102A nuclear accumulation. (a) 2.3 x 105 MCF7 breast carcinoma cells were seeded on a 24 x 24mm steryl coverglass in 60mm dishes and were transiently transfected with FlagYB-1S102A plasmid alone (0,3 µg, left panel) or with CA-Akt1 plasmid (1 µg, right panel), as indicated in figure. Cells were fixed with PFA and processed for indirect immunofluorescent with rabbit anti-flag antibody and secondary Cy3-coniugated rabbit antibodies (green). DAPI counterstain (blue) indicates the location of nuclei and merged images were generated using Adobe Photoshop CS3 software. **(b)** MCF7 cells were seeded at 60% confluency (2.3 x 105) on 24 x 24 mm sterile coverglasses placed in 60 mm dishes and transiently transfected with 1 µg of Flag YB1-S102A and a fixed amount of pcDNA3/ΔNp63α expression vectors (1µg) expression vectors. Cells were fixed and subjected to double indirect immunofluorescence as indicated in Materials and Methods, using anti-Flag antibody and secondary anti-rabbit Fitch-conjugated (green). p63 was detected using anti-p63 antibodies and secondary anti-mouse Cy3-conjugated (red). Merge between DAPI and Flag-YB1 S102A are indicated. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei.

Finally, I observed that compared to the wild type or the FlagYB-1S102A protein, the phospho-mimetic FlagYB-1S102D mutant was expressed at lower level (Figure 19). This suggested that instead to promote nuclear accumulation, phosphorylation of Serine 102 by AKT was affecting YB-1 protein stability. Accordingly, as detected by western blot analysis, CA-AKT expression reduced the abundance of both wild type and FlagYB-1S102A protein thereby suggesting that AKT can decrease YB-1 stability by phosphorylating multiple phospho-acceptor sites of YB-1. Consistently, the fluorescent signal corresponding to the FlagYB-1S102D mutant was hardly detectable in cells transfected with CA-AKT (Figure19). Conversely, this mutant gave a strong nuclear fluorescent signals in ΔNp63α positive cells.

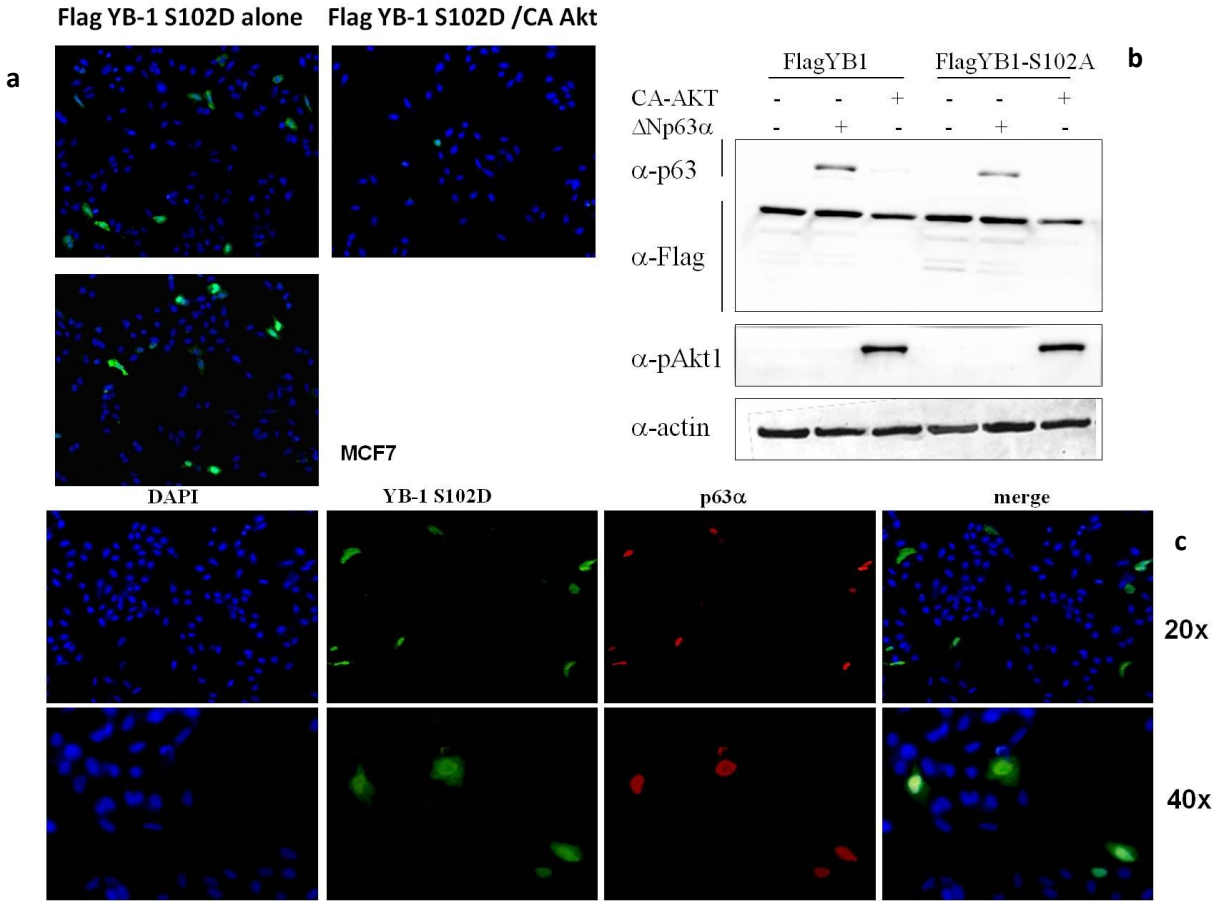


Figura 19: Akt activation does not promote, by itself, YB1S102D nuclear accumulation. 2.3 x 105 MCF7 breast carcinoma cells were seeded on a 24 x 24mm steryl coverglass in 60mm dishes and were transiently transfected with FlagYB-1S102D plasmid alone (0,3 µg, left panel) or with CA-Akt1 plasmid (1 µg, right panel), as indicated in figure. Cells were fixed with PFA and processed for indirect immunofluorescent with rabbit anti-flag antibody and secondary Cy3-coniugated rabbit antibodies (green). DAPI counterstain (blue) indicates the location of nuclei and merged images were generated using Adobe Photoshop CS3 software. **(b)** MCF7 cells were seeded at 60% confluency (2.3 x 105) in 60mm dishes and transfected with wild type FlagYB-1 or FlagYB-1S102A (0,3 µg) plasmid alone or with CA-Akt1 (1 µg) or ΔNp63α plasmid (1 µg). Total cells extracts, separated in 10% polyacrilammide gel, were analyzed by immunoblot using the indicated antibodies. Actin was used as loading control. **(c)** MCF7 cells were seeded at 60% confluency (2.3 x 105) on 24 x 24 mm placed in 60 mm dishes and

transiently transfected with 1 µg of Flag YB1-S102D and a fixed amount of pcDNA3/ΔNp63α expression vectors (1µg). Cells were fixed and subjected to double indirect immunofluorescence, using anti-Flag antibody and secondary anti-rabbit Fitch-conjugated (green). p63 was detected using anti-p63 antibodies and secondary anti-mouse Cy3-conjugated (red). Merge between DAPI and Flag-YB1 S102A are indicated. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei.

3.6 ΔNp63α influences YB1-protein turnover

By immunoblot analyses I confirmed that the expression level of the phospho-mimetic mutant YB-1S102D (50 kD) was significantly lower compared to the wild type and YB-1S102A proteins. However, ΔNp63α enforced expression caused a 2.28 folds increase of YB-1S102D protein abundance (Figure 20b) with a similar increase of its nuclear-cytoplasmic ratio (Figure 20 c and d).

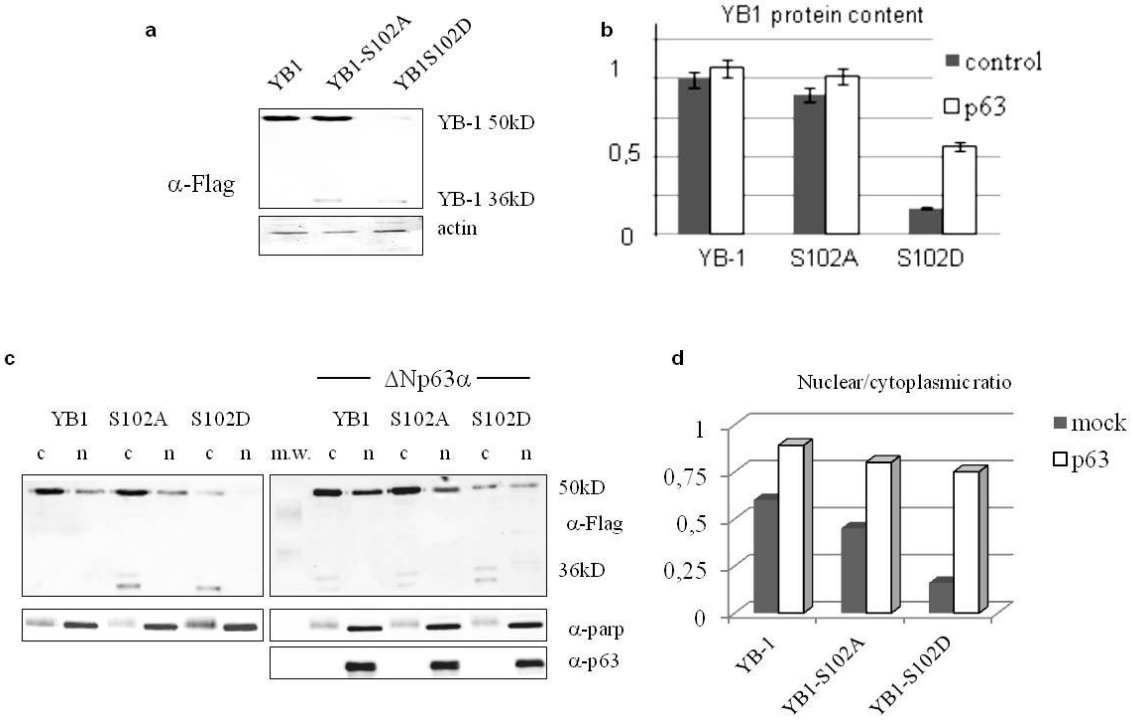


Figura 20 ΔNp63α increase YB-1 nuclear fraction. (a) MCF7 cells, seeded at 60% confluency (2.3 x 105) in 60mm dishes, were transiently transfected with vector expressing wild type FlagYB-1, S102A or S102D mutants (0.3 µg each). Equal aliquots of total cell lysates (10 µg) were separated in 10% SDS-PAGE and subjected to immunoblot with anti-Flag polyclonal antibody. Actin was used as a loading control **(b)** Plots showing the expression of total wild type, S102A or S102D FlagYB-1 proteins with or without p63. Data were obtained by densitometry scanning of immunoblot revealed with anti-Flag antibodies and normalized to actin expression. Values are mean±S.D. of three independent experiments **(c)** 5 x 10^6 MCF7 cells, seeded in 100mm dishes, were electropored with 2 µg of each vector expressing wild type FlagYB-1, S102A or S102D mutants with or without 4 µg of ΔNp63α plasmid. At 24h from transfection nuclear and cytoplasmatic extracts were prepared as indicated in Materials and Methods, analysed by 10% SDS-PAGE and subjected to immunoblot with the indicated antibodies. Total Akt and PARP antibodies were used as cytoplasmic/nuclear loading and quality control. **(d)** Plots showing the average value of YB-1 protein nuclear/cytoplasmic ratio obtained by densitometric scanning of immunoblot with anti-Flag antibodies. Both cytoplasmic and nuclear extracts were first normalized for

actin loading control. For each value the average of five different exposures was calculated and each value was normalized against total AKT and PARP for cytoplasmic and nuclear extracts, respectively.

These preliminary observations prompted us to further investigate about the effect of ΔNp63α on YB-1 protein stability. Therefore, we measured YB-1 protein half-life in mock and ΔNp63α transfected MDA-MB-231 cells, by treating cells with cycloheximide (CHX). As shown in Figure 21, the rate of decay of endogenous YB1 follows a biphasic pattern: an initial phase in which YB1 decays rapidly (half-life of about 2 hrs) is followed by a latter phase in which the YB1 protein level remains substantially unchanged.
Next, I measured YB-1 protein half-life in ΔNp63α transfected cells and I found that the initial phase, characterized by rapid YB-1 protein degradation, was strongly reduced.
In agreement with our results with transfected p63, the rate of decay of endogenous YB-1 in HaCaT cells, expressing endogenous ΔNp63α, was remarkably slower than in H1299 cells lacking p63, thereby indicating that it was not an artifact of transient ΔNp63α over-expression (Figure 21b).
Finally, I measured YB-1 protein half-life, by cycloheximide decay, in nuclear and cytoplasmic fractionated extracts. Compared to cytoplasmic YB1, the nuclear protein had a significantly reduced rate of turnover (Figure 21c and d).
All together, these observations suggest that the nuclear pool of YB-1 protein is more stable than the cytoplasmic one and ΔNp63α appears to increase the nuclear, slow degrading pool of YB-1.

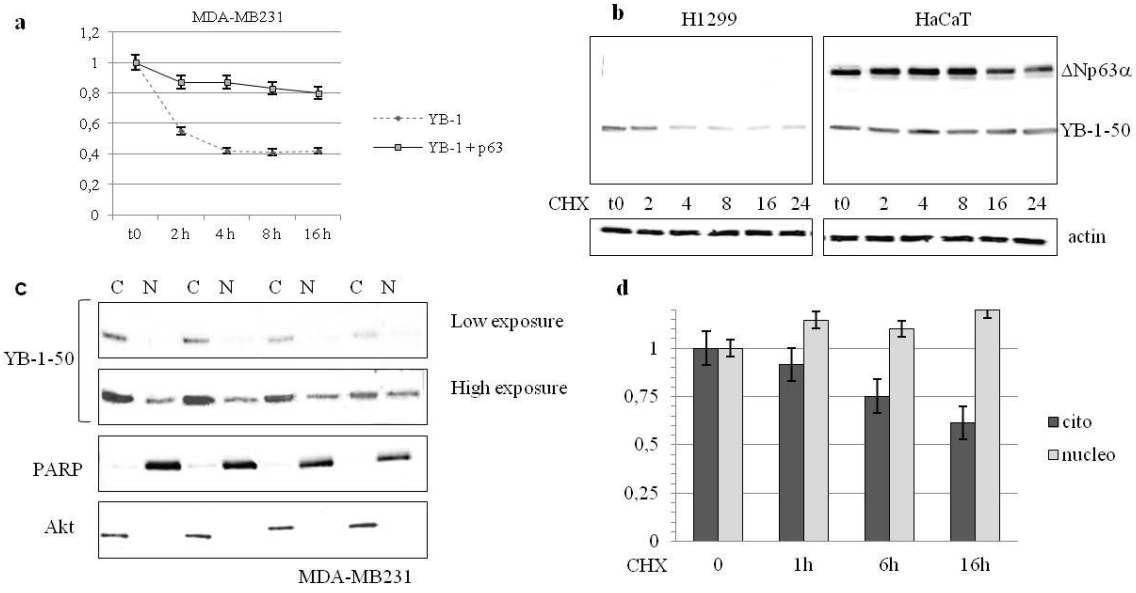


Figura 6 ΔNp63α influences YB-1 protein turnover. (a) At 16 hrs, 20 μM cycloheximide (CHX) was added to mock and ΔNp63α transfected MDA-MB-231 cells. At the indicated times, cell were harvested and equal amounts of protein extracts were subjected to immunoblot with anti-YB-1 antibodies. Data from densitometric scanning were normalized against actin and plotted (YB-1, dotted line; YB-1 + p63, continuos line). Standard deviations from 3 independent experiments are indicated by error bars. **(b)** H1299 cells (devoid of p63, left panel) and HaCaT cells (expressing abundant endogenous ΔNp63α, right panel) were seeded at 60% confluence (2.3 x 105) in 60mm dishes and treated with 20 μM CHX, for the indicated times. Equal amounts of extracts were subjected to immunoblot with anti YB-1 and anti p63 antibodies. Actin was used as loading control. **(c)** 3 x 106 MDA-MB-231 cells were seeded in 100mm dishes (60% confluency) and treated with 20 μM cycloheximide for the indicated times. Nuclear and cytoplasmic fractionated extracts (2,5 μg) were separated by SDS-PAGE and subjected to immunoblot with anti-YB-1 antibody. Total Akt and PARP antibodies were used as loading and quality control of cytoplasmatic or nuclear extracts, respectively. **(d)** Plots showing the rate of decay of cytoplasmic and nuclear YB-1 as obtained by densitometry scanning. Data were obtained as the average of five different exposures and each value was normalized to the appropriate loading control (Total-Akt or PARP). The relative values for cytoplasmatic and nuclear YB-1 protein distribution were calculated by placing equal to one the expression of cytoplasmatic and nuclear YB-1 at time 0. The data are from three independent experiments and the standard deviation are indicated by error bars.

3.7 ΔNp63α and YB-1 nuclear functions

YB-1 directly interacts and activates the promoter of PIK3CA gene, encoding the p110α catalytic subunit of phosphatidylinositolo-3-kinase, thereby increasing PI3K/Akt activity (Astanehe et al., 2009). Accordingly, YB-1 depletion in MDA-MB-231 cells resulted in a reduction of Akt1 phosphorylation (Figure 22, left).

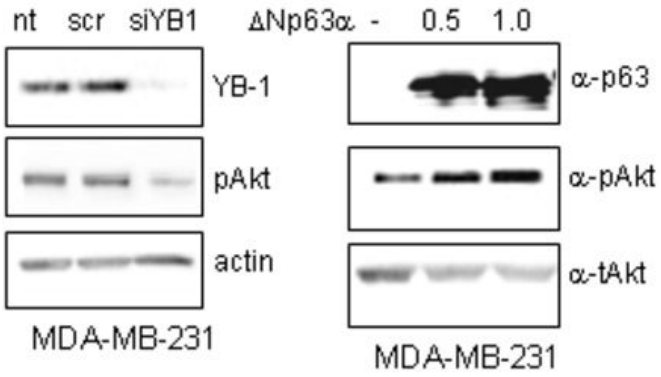


Figura 7 ΔNp63α controls YB-1 nuclear function. (left) MDA-MB-231 cells were transfected with 25nM of siRNA to YB-1 and scrambled control or (right) with increasing amounts of pcDNA3/ΔNp63α encoding plasmid. 48hr after transfection, cells were lysate and 20μg of total extract were subjected to Western Blot analysis and immunorevealed with anti-total AKT, pAKT or anti-YB-1.

As we noticed that enforced expression of ΔNp63α caused a dose dependent enhancement of Akt1 phosphorylation (Figure 22, right), we monitored the response of PIK3CA gene promoter to ΔNp63α, by luciferase assays (see materials and methods).

As shown in Figure 23, ΔNp63α enforced expression caused a significant increase of the PIK3CA promoter activity that was attenuated by YB-1 knock-down.

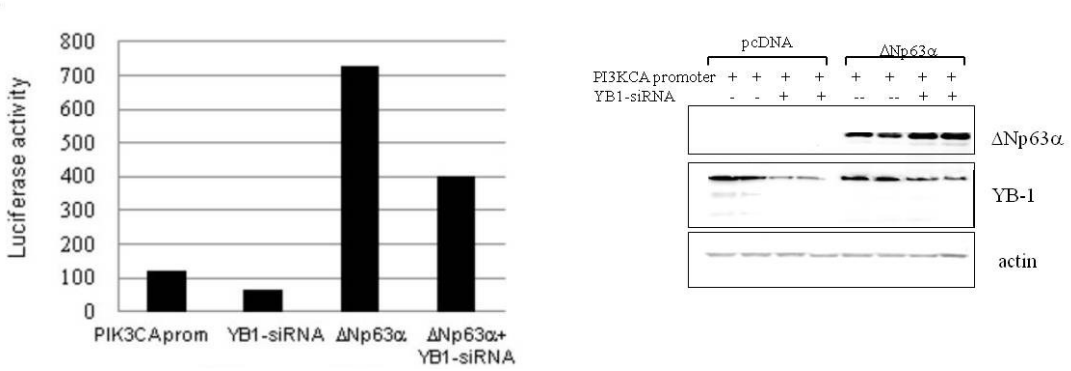


Figura 8 MDA-MB-231 cells were transfected with 1µg of luciferase reporter construct containing PIK3CA promoter and luciferase activity was measured upon YB-1siRNA, ΔNp63α or ΔNp63α and YB-1siRNA transfection. The results are reported as the average of at least three independent experimental points (left panel). Aliquots of duplicate samples assayed by Luciferase activity (20 µg) were subjected to immunoblot with the indicated antibodies (right panel)

Next, we performed Chromatin IP (ChIP) in MDA-MB-231 cells transfected with Flag-YB1 with or without ΔNp63α to evaluate the binding of YB-1 to the PIK3CA promoter, a well known YB-1 target (Astanehe et al., 2009). As shown in Fig 7d, ΔNp63α dramatically increased the binding of Flag-YB1 to the PIK3CA promoter. Next, ChIP was performed using antip63 (4A4) antibodies in cells transfected with ΔNp63α or ΔNp63γ and we found that ΔNp63α, itself, binds to the PIK3CA promoter whereas ΔNp63γ failed to do so (Figure 7e).

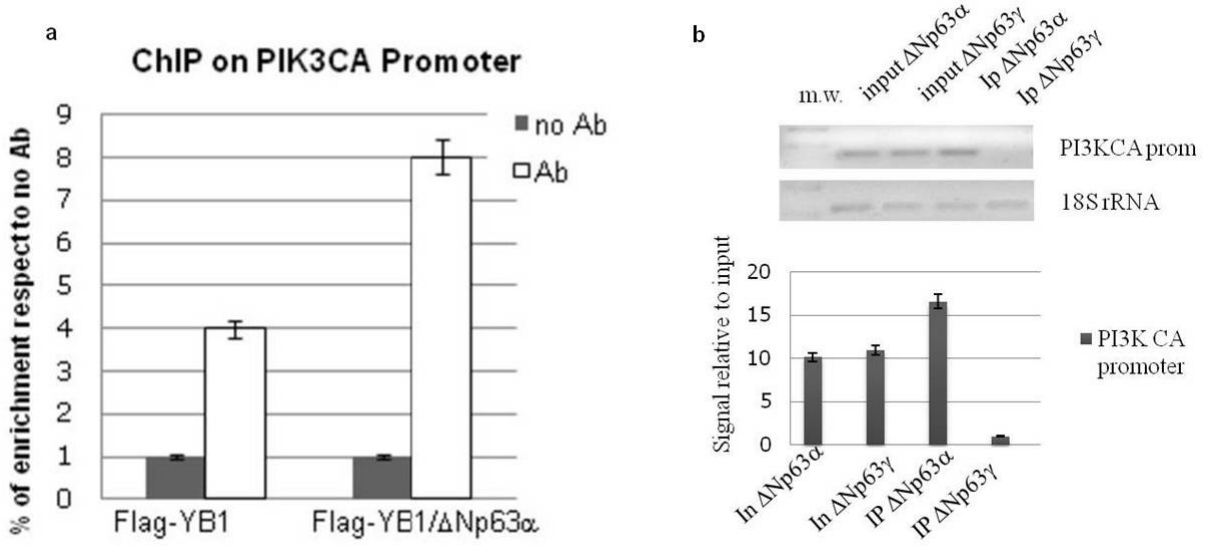


Figura 24 (a) MDA-MB-231 cells were seeded at 60% confluency (1.2 x 106) in 100mM dishes and transiently transfected with 3XFlag-YB-1 (5 µg) construct with or without ΔNp63α (2.5 µg) expression vector. The cells were cross-linked with formaldehyde and DNA/protein complexes were immunoprecipitated with anti-Flag antibody or irrelevant IG g antibody as negative control. The DNA immunoprecipates were analysed by qPCR using PIK3CA or GAPDH promoter oligos. qRTPCR results were analized with the ΔΔCT method and expressed as fold of enrichment respect to the IG g control samples. of each input. Values are represented as the mean of three independent experiments. **(b)** MDA-MB-231 cells were seeded at 60% confluency (1.2 x 106) in 100mM dishes and transiently transfected with ΔNp63α or ΔNp63γ plasmid (5 µg). After formaldehide crosslinking, the DNA/proteins complexes were immunoprecipitated with anti-p63 (4A4) antibody. Immunoprecipitated DNA was PCR amplified with PIK3CA promoter oligos and 18S oligos. Input DNA (3% of total immunoprecipitate) was amplified to demonstrate that primers produced the expected PCR products (upper panels). M.W. molecular weight DNA ladder. The data obtained from the ChIp assay were measured by densitometry and are presented as

3.8 ΔNp63α affects YB-1 cytoplasmic functions

Cytoplasmic localization of YB-1 was associated with binding and translational activation of Snail1 mRNA (Evdokimova et al., 2009). By western blot experiment and semiquantitative RT-PCR, I have shown that ΔNp63α, but not ΔNp63γ, reduced Snail1 protein level without changing the level of Snail1 transcript (Figure 25 a and b). Interestingly, the level of Twist specific transcript, instead, appeared to be reduced by ΔNp63α expression (Figure 25 b).

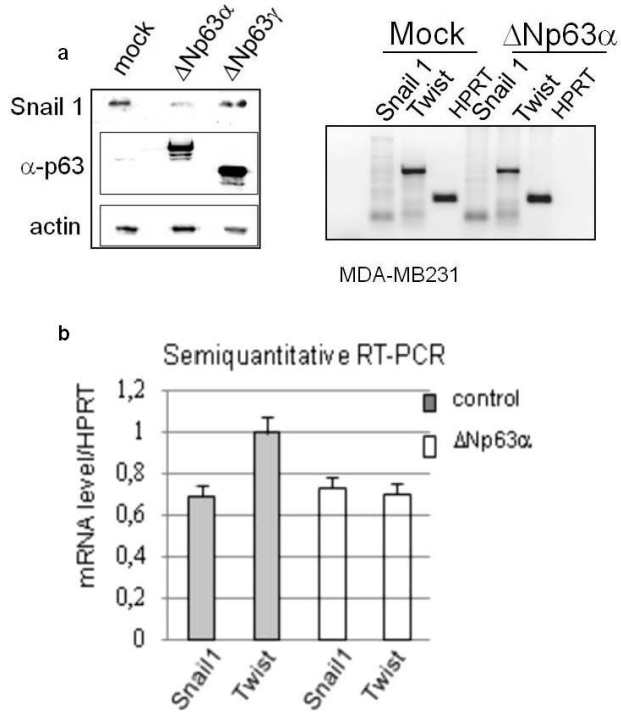


Figura 9 ΔNp63α affects YB-1 cytoplasmic functions. (left panel) MDA-MB-231 cells were transfected with an empty vector (mock) or a fixed amount (1µg in 60mm dishes) of pcDNA3/ΔNp63α or pcDNA3/ΔNp63γ expression vector. 24 hr after transfection, cells were harvested and total extracts were prepared. 20µg of each extract were loaded on SDS-PAGE and subjected to immunoblot with the indicated antibodies. **(right panel)** MDA-MB-231 cells were transfected with an empty vector or pcDNA3/ΔNp63α encoding plasmid. The expression level of ΔNp63α was checked by immunoblot (Figure 14a, left panel). 24hr after transfection total RNA was purified and retrotranscribed as described in Materials and Methods. PCR was performed with primers designed to specifically amplify Snail1, Twist or HPRT transcripts (see Materials and Methods). **(b)** Plot showing the level of Snail1 and Twist transcripts, normalized respect to HPRT. Values are the mean of 3 independent experiments.

Next, I performed RNA-Immunoprecipitation to determine the amount of Snail1 mRNA bound to YB-1, and I found that it was significantly reduced in presence of ΔNp63α (Figure 26).
Since Snail1 is known to be directly implicated in E-cadherin repression (Peirò et al., 2006), I checked the expression level of E-cadherin upon ΔNp63α enforced expression in MCF7 cells that express detectable levels of both E-cadherin and Snail1. As shown in Figure 26 (right panel), ΔNp63α caused a dose-dependent increase of E-cadherin that was associated with a parallel reduction of Snail1.

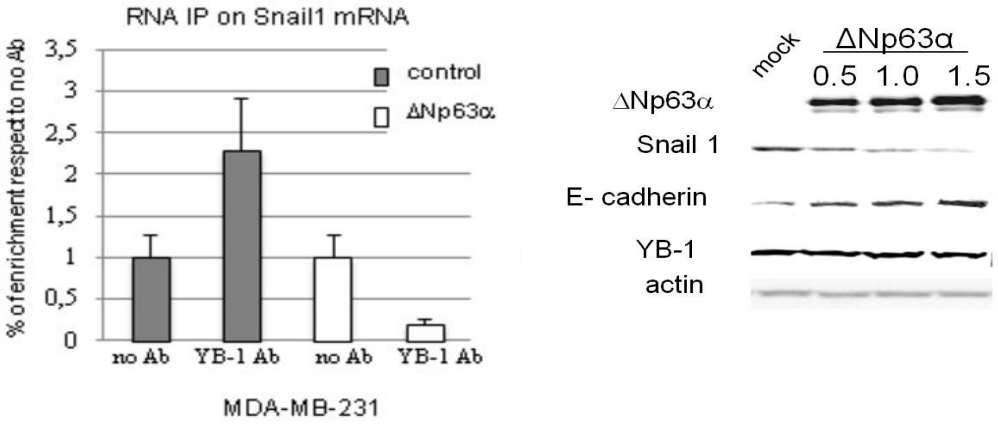


Figura 26 (left) Nuclear extracts from ΔNp63α transfected MDA-MB-231 cells were immunoprecipitated with anti-YB1 antibody. After reverse cross-linking the YB1-bound RNA was purified, retrotranscribed and subject to qRT-PCR analysis using oligonucleotides designed to specifically amplify Snail1 transcript. Plot represents the % of enrichment of Snail1 transcript normalized as indicated in Materials and Methods. 1/50 of the input extract was loaded as control. **(right)** MCF-7 cells were transfected with an empty vector or the indicated amount of pcDNA3/ΔNp63α plasmid. 24hr later, cell extracts were prepared and 30μg of each were loaded on SDS-PAGE and subjected to immunoblot with the indicated antibodies. Actin was used as loading control.

Finally, because it was reported that cytoplasmic YB-1 inhibits translation of its own mRNA, by specific interaction with the 3'UTR (Skabkina et al., 2005), I tested the effect of ΔNp63α on the ability of YB-1 to bind to its own transcript, by RNA immunoprecipitation. Briefly, YB1-bound cytoplasmic RNA was immunoprecipitated with antibodies against YB-1. As shown in Figure 27, binding of YB-1 to its own transcript was substantially reduced in MDA-MB-231 cells expressing ΔNp63α.

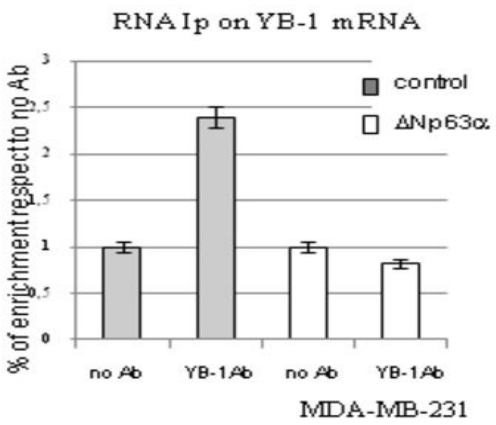


Figura 27 YB-1- bound RNA was obtained from mock or ΔNp63α transfected MDA-MB-231 cells by RNA immunoprecipitation. RNA was retrotranscribed and subjected to qRT-PCR using oligonucleotides designed to specifically amplify YB-1 transcript. Plot represents the % of enrichment of YB-1 transcript normalized respect to no-Antibodies sample.

3.9 Effects of ΔNp63α and YB1 on cell migration

Recent studies have provided convincing evidence that YB-1 up-regulation drives epithelial to mesenchimal transition (EMT) that is associated with reduced proliferation, increased cell motility and invasiveness (Evdokimova et al., 2009). Since ΔNp63α affects YB-1-dependent transcription of pro-migratory genes such as Snail1, we hypothesize that it should also restrain the migratory ability of YB1 overexpressing cells. This aspect was addressed with the collaboration of prof. Netti group using genetically modified TetOn-H1299 cells expressing ΔNp63α upon Doxycycline (Dox) addition (Figure 28). Cells were transfected with GFP or GFP-YB1 plasmid and unfluorescent and fluorescent cells were tracked separately.
Cells migrated describing trajectories that are randomly distributed in plane, as depicted in plots of Figure 28b. Root mean square speed (S) and persistence time (P) were computed from cell tracks and are a measure of the frequency of cell steps and of the minimum time that is necessary for a cell to significantly change direction. In Dox free medium (-Dox), GFP-YB1 expressing cells were characterized by a significantly higher S and lower persistence (0.69 ± 0.04 μm/min and 8.4 ± 0.5 min) than cells expressing GFP alone (0.54 ± 0.05 μm/min and 15 ± 2.1 min). Remarkably, in Dox supplemented medium (+ Dox), GFP-YB1 expressing cells exhibited a S and P values (0.62 ± 0.5 μm/min and 9.2 ± 1.0 min), which were not significantly different from those of cells transfected with GFP alone (0.68 ± 0.05 μm/min and 8.6 ± 0.9 min). We also noticed that unfluorescent cells were characterized by the highest S

values suggesting that expression of the Green Fluorescent Protein alters cell migration to some extent (data not shown). However, S of unfluorescent cells with or without Dox was comparable.

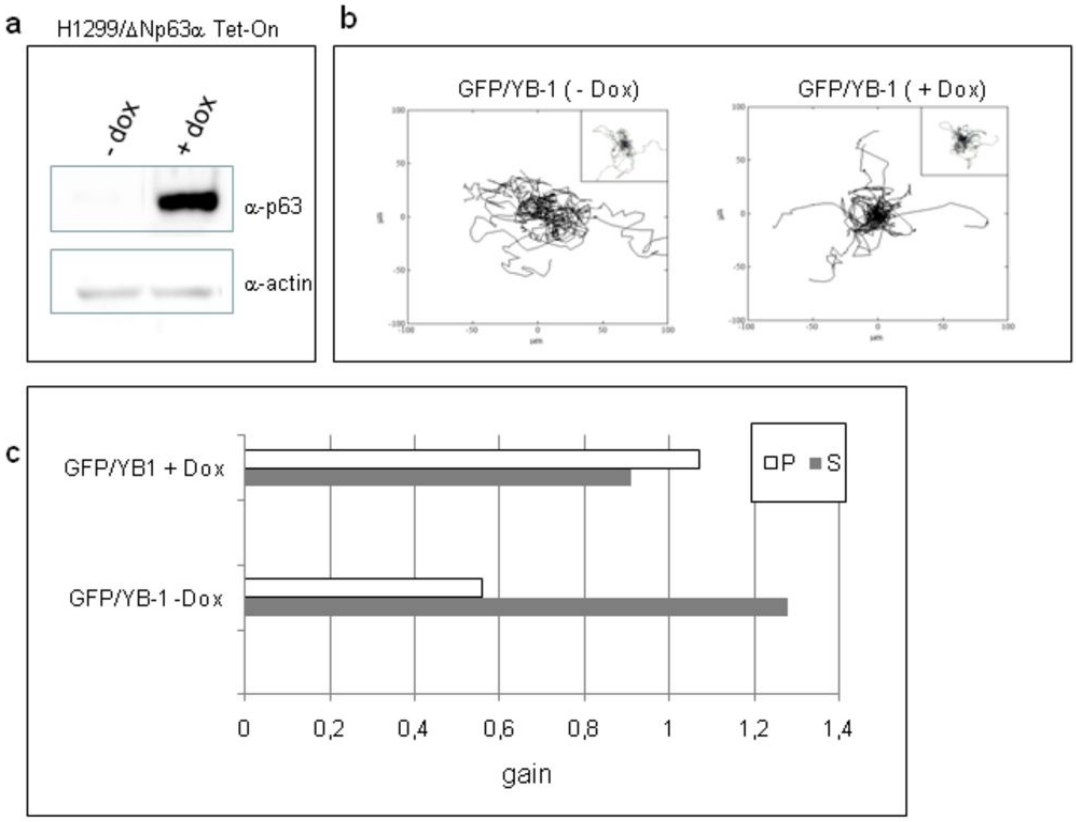


Figura 28 ΔNp63α counteracts YB-1 induced enhancement of cell migration. (a) Immunoblot analysis of ΔNp63α in H1299 cells treated (+ Dox) or not (- Dox) with Doxicycline. After migration analysis, cells extracts were prepared and subjected to immunoblot with the indicated antibodies to monitor ΔNp63α induced expression. **(b)** Trajectories of GFP/YB-1 or GFP (insets) transfected cells. Inducible H1299/ΔNp63α cells were transfected with a fixed amount of GFPYB1(1μg) or GFP empty vector (1μg). 4hr after transfection, 2 μg/ml of doxycycline was added to induce ΔNp63α expression and 16 hr later uninduced (-Dox) and induced (+Dox) cells were tracked with optical microscopy. Plots represent data from the following number of cell (YB-1/-dox 79; YB-1/+dox 69; control/-dox 44; control/+dox 49). **(c)** Bar chart of speed (S; grey bars) and persistence ratios (P; white bars). The ratios are computed by dividing the population average values of speed and persistence for H1299 cells treated (+ Dox) or not (- Dox) with Doxicycline.

3.9.1 Expression of p63α and YB-1 in resting and regenerating cornea

We have previously shown that YB-1 and ΔNp63α are co- I have previously observed that YB-1 and ΔNp63α are co-expressed in proliferating keratinocytes and both are downregulated during calcium-induced differentiation.
In collaboration with Dr. Di Iorio of the "Eye bank foundation", Zelarino, Venezia I have investigated the role of YB-1 and p63 in normal and regenerating corneal tissues. We have chosen the cornea because it is an interesting model to investigate the expression of both p63 and YB-1 markers during differentiation. Therefore, we speculated that a functional interplay between these two proteins may occur in basal proliferating keratinocytes of adult tissues or in regenerating epithelia, for instance after mechanical injury. The corneal regenerating epithelium provides a suitable model to follow the expression of YB-1 and p63 during transition between epithelial stem cells and terminally differentiated keratinocytes. Specifically, corneal stem cells are segregated in the basal layer of the limbus. When activated by mechanical injury, they produce Transit-Amplifying (TA) cells that divide only a few times during their migration from the limbus to the central cornea where they terminally differentiate (Di Iorio et al., 2005). In unperturbed corneas, p63 is present only in patches of basal limbal stem cells while it is undetectable in the differentiated central corneal epithelium (Di Iorio et al., 2005). YB-1, instead, is expressed in limbus as well as in central cornea and is prevalently cytoplasmic (Figure 29a and b). In limbus of resting corneas, a lot of cells show overlap between ΔNp63α and YB-1 (Figure 29a). Overlapping between YB-1 staining and CK12, a marker of corneal cell differentiation indicates that, unlike p63, cytoplasmic YB1 expression persists in differentiating corneal cells (Figure 29b). In activated cornea, where p63 decorates the nuclei of basal and suprabasal layers of limbal and central cornea, p63 and YB-1 colocalization was frequently detected and particularly in cells moving from the basal towards the suprabasal layers (Figure 29c). Of interest, at higher magnifications (63X), YB-1 signal was clearly localized either in the nucleus (highlighted with number) or in the cytoplasm, thus confirming previous data showing that YB-1has the capacity for continuous shuttling between the cytoplasm and the nucleus (Figure 30a).
The expression of p63 is strongly correlated to cell size and it is expressed in a small amount of undifferentiated limbal stem

cells (holoclones) and progressively decreases during clonal conversion from holoclone to paraclone and serial in vitro propagation, in which a consistent increase of cell size is usually observed (Di Iorio et al., 2005).
In samples of corneal primary cultures, corneal stem cells, the smallest ones, express p63 and YB-1 abundantly (Figure 30b, left panel), but after serial cultivation (after the fifth passage), the large and terminally differentiated corneal keratinocytes appeared to be devoid of both p63 and YB1 specific staining (Figure 30b,right panel, yellow asteriks).

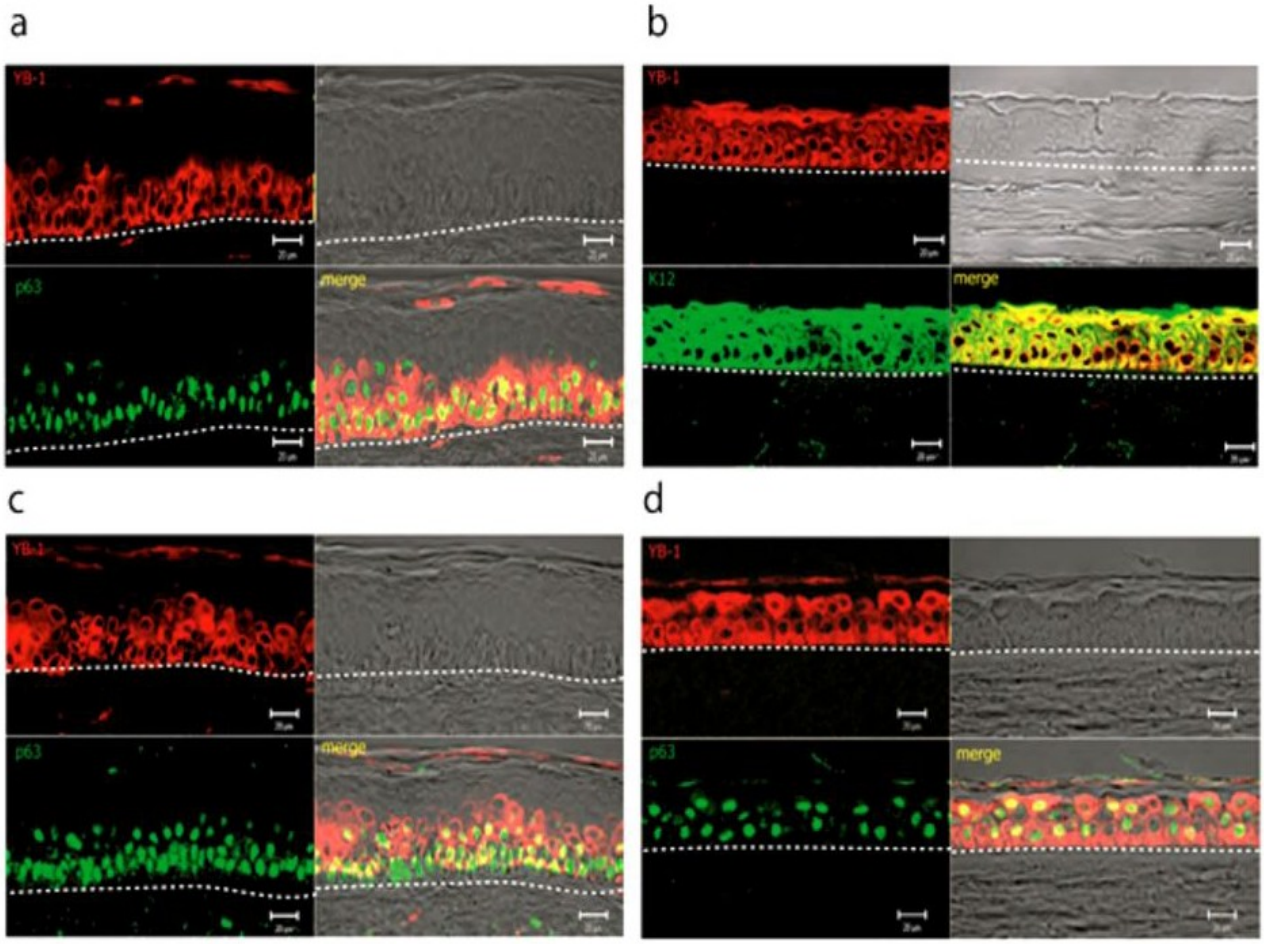


Figura 10 Expression of p63 and YB-1 in cornea. Double immunofluorescence of YB-1 *(red)* with **(a)** p63 (green) on sections of resting limbus and with **(b)** cK12 (green) on sections of resting cornea. p63 and YB-1 were coexpressed by a discrete number of basal cells (merge frame), but YB1 expression persisted in differentiating corneal cells, as shown by overlap con cK12. In activated limbus **(c)** and cornea **(d)**, where p63 decorates the nuclei of basal and suprabasal layers, p63 and YB-1 colocalization was frequently detectable and particularly in cells moving from the basal towards the suprabasal layer.

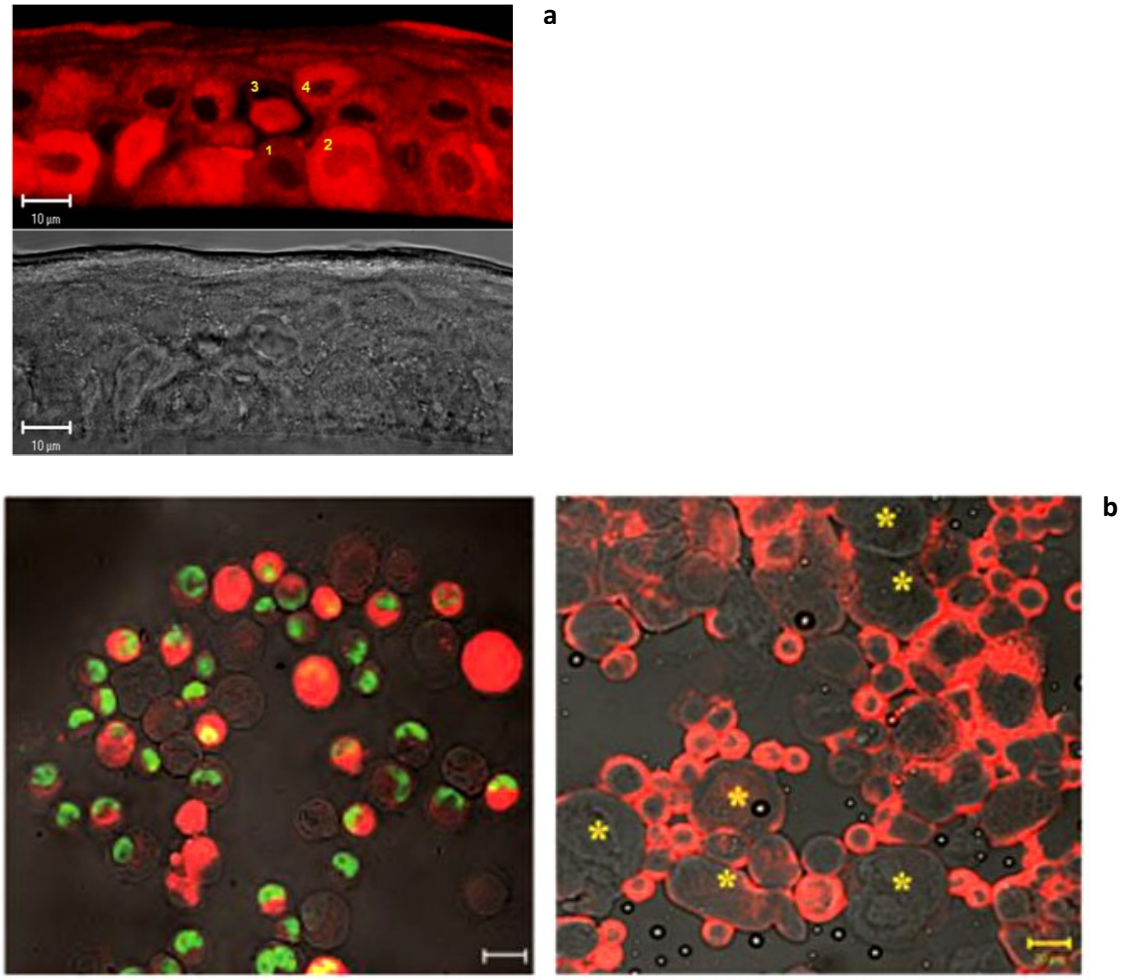


Figura 11: (a) Higher magnification (63X) showed nuclear localization of YB-1 in some cells, indicated by yellow asterisks. **(b)** Cytological detection of YB-1 (red) and p63 (green) in corneal primary cultures: note the high percentage (60%–70%) of cells in which p63 and YB-1 are coexpressed. (right panel) When corneal keratocytes were serially propagated in vitro, YB-1 expression was definitely lost in large and terminally differentiated cells indicated by asterisks.

4. Discussion

My work unveils a novel protein-protein association involving ΔNp63α and YB-1 and a mechanism through which a factor essential for epithelial development and differentiation can control the activity of an oncoprotein. By in vitro and in vivo protein-protein interaction assays we have unequivocally demonstrated that the α-specific C-terminal region of p63 and the 36 kDa Nterminal fragment YB-1 are involved in such physical association. In malignant cells, overexpression and nuclear localization of YB1 is correlated with multidrugresistance and hyper-proliferation (Janz et al., 2002). In normal cells, instead, the majority of YB1 protein localizes to the cytoplasm, with a minor pool located into the nucleus.

My data show that ΔNp63α enforced expression promotes YB-1 nuclear accumulation and Akt phosphorylation (Sabbisetti et al., 2009). In principle, p63-driven YB-1 nuclear accumulation could be considered as a consequence of Akt activation. However, as we have shown that exogenous expression of constitutively activated Akt (CA-Akt) did not result in YB-1 nuclear translocation, the physical interaction with ΔNp63α seems to be necessary for YB-1 nuclear targeting. Expression of CA-Akt, instead, appeared to cause a moderate reduction of the level of cytoplasmic YB-1 and its preferential re-distribution from a diffuse cytoplasmic to a perinuclear localization. These observations are in line with previous findings showing that PI3K/Akt signaling activation, instead of causing YB-1 nuclear localization, impairs the binding of YB-1 to cytoplasmic transcripts releasing their translational repression (Evdokimova et al, 2006). Furthermore, the phosphorylation defective YB-1Ser102A protein, as the wild type and Ser102D mutant protein, colocalizes with ΔNp63α into the nuclear compartment thus indicating that disruption of the major phosphoacceptor site on YB-1 does not prevent YB1 nuclear translocation by ΔNp63α.

Furthermore, we present evidences that Akt activation, rather than promoting YB-1 nuclear translocation, plays a role in the control of YB-1 protein turnover. In fact, we observed that wild type YB-1 as well as YB-1S102A protein level was reduced by CA-Akt thus suggesting that Akt targets multiple phospho-acceptor sites, including Serine 102, thereby reducing YB-1 protein stability. According to this hypothesis, we found that the phospho-mimetic YB1-S102D mutant was expressed at very low level. Moreover, by cycloheximide decay experiments, we have observed that nuclear YB1 protein displays a slow rate of turnover when compared to its cytoplasmic fraction.

Therefore, we suggest that ΔNp63α, by targeting YB-1 into the nucleus, increases the slow degrading component of YB-1. YB-1 and ΔNp63α are both transcription factors, and a considerable number of their targets have already been identified (Jurchott et al, 2006). However, an intriguing point is whether the association between YB-1 and ΔNp63α can drive the complex on particular promoters.

Remarkably, we show that the PIK3CA gene, a well characterized YB-1 target gene, is also regulated in vivo by ΔNp63α. ChIP analyses demonstrate that ΔNp63α binds to the PIK3CA promoter; it remains to be addressed whether the two proteins bind separately or as a complex to this promoter.

Into the nuclear compartment, ΔNp63α and YB1 support PI3K/Akt signaling activation thereby increasing cell survival. On the other hand, Akt is known to blunt tumor cell invasion by blocking breast cancer cell motility through the inhibition of NFAT transcriptional activity (Yoeli-Lerner et al, 2005). Here we report that YB1 overexpressing tumor cells increase their speed by a factor 1.3 while decreasing persistence time by approximately 40%.

Remarkably, we found that ΔNp63α expression restores a normal migratory behaviour with reduction in speed and simultaneous increase of persistence (figure 12c). Moreover, we have shown that ΔNp63α increases E-cadherin and restrains YB-1-dependent expression of pro-migratory genes such as Snail1 and Twist, thus counteracting cancer metastatic spread (Higashikawa et al., 2007). All together, our observations support the idea that ΔNp63α-YB1 association, in primary tumors, can act as a pro-survival mechanism while attenuating cell motility possibly explaining why ΔNp63α expression is selected against in highly metastatic carcinoma cells (Graziano et al., 2011). Such speculations however ought to be further investigated with invasion experiments performed in more “physiologic” environments.

Finally, although the generally accepted view is that YB-1 is preferentially expressed in cancer we have demonstrated that YB-1 is abundantly expressed in normal tissues such as primary keratinocytes and corneal epithelium and, in particular, in cells having a proliferating potential.

Remarkably, frequent overlapping between p63 and YB-1 is detected in injury-activated corneas when stem cells are induced to develop into Transient Amplifying (TA) that divide a few times to regenerate the tissue and move to the suprabasal compartment. An intriguing possibility is that YB- 1 and ΔNp63α nuclear localization might be transiently induced by mechanical stress to promote resistance to anoikis, a cell death phenomenon involving epithelial cells induced by detachment

from the extracellular matrix, an aspect that can have important implications not only for cancer, but also for developmental and differentiation processes that are characterized by a precisely regulated balance between cell proliferation, migration and death.

5. Materials and Methods

Plasmids

cDNA encoding human Flag-tagged YB1, YB1-S102A and YB1-S102D were provided by Dr. Sandra Dunn (Research Institute for Children's and Women's Health, Vancouver, British Columbia, Canada). PIK3CA luciferase plasmid was provided by Dr Arezoo Astanehe (Research Institute for Children's and Women's Health, Vancouver, British Columbia, Canada). cDNA encoding human ΔNp63α and ΔNp63γ were previously described (Di Costanzo et al., 2009). For bacterial expression, ΔNp63γ and ΔNp63α cDNAs were inserted in pRSETA vector in XhoI/ClaI and XhoI/XbaI (filled) sites, respectively. GFP and YB1-GFP vectors were provided by Dr. Paul R. Mertens (University Hospital Aachen, Germany). PET-YB1 was from Dr. Jill Gershan (Medical College of Wisconsin, Milwaukee, Wisconsin). CA-Akt was a gift from Dr. G. Condorelli (IRCCS Ospedale San Raffaele, Milano).

Cell lines, transfection and antibodies.

MDA-MB-231, MCF7, HaCaT and H1299 cells were obtained from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO2. Dox-inducible Tet-On H1299/ΔNp63α cells were previously described (Lo Iacono et al., 2005). Lipofections were performed with Lipofectammine (Invitrogen), according to the manufacturer's recommendation. YB1 transient silencing was carried out with ON-TARGET plus SMART pool YB1-siRNA (Dharmacon) and RNAiMAX reagent (Invitrogen). Anti-p63 (4A4) and anti-actin (1-19) were from Santa Cruz (Biotechnology Inc.), anti-FLAG M2 and anti-GFP (sc-8334) were from Sigma-Aldrich (Germany). PARP, Akt, pAkt (Ser473) and YB1 antibodies were from Cell Signaling Technology (Beverly, Massachussets). Anti-GFP antibody was from (Roche Applied Science). Mouse monoclonal E-cadherin (ab1416) and Snail1 antibodies were from Abcam (Cambridge, UK). Cy3-conjugated anti-mouse IgG was from Jackson (ImmunoResearch Laboratory, Inc, Pennsylvania). Doxycycline

was from Clonetech (Palo Alto, California). MG132 was from Calbiochem (San Diego, California). Cycloheximide was from Sigma (Sigma-Aldrich, Germany).

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed with chromatin from human MDA-MB-231 cells transfected with ΔNp63γ, ΔNp63α and/or FlagYB1, according to Radoja et al. (Radoja et al., 2007). Real Time PCR was performed with the 7500 Applied Biosystems apparatus and Syber Green MasterMix (Applied Biosystems) using the following oligonucleotides:
PIK3CA-Forw CCCCCGAACTAATCTCGTTT
PIK3CA-Rev TGAGGGTGTTGTGTCATCCT

Immunoblot analyses and coimmunoprecipitation

Immunoblots and coimmunoprecipitations were performed as previously described (Di Costanzo et al., 2009). To detect p63/YB-1 interaction in H1299 cells, 5.0 ×105 cells were plated in 60 mm dishes and transfected with 1μg of ΔNp63α or ΔNp63γ plasmids. For coimmuprecipitations, whole cell extracts, precleared with 30 μl of protein A-agarose (50% slurry; Roche, Manheim, Germany), were incubated overnight at 4°C with anti-YB-1(3 μg), anti-p63 (2 μg) or anti-Flag (3 μg).

Far-western

Far-western assays were conducted according to the protocol described by Cui S et al. 2004 Appropriate amounts of ΔNp63α or ΔNp63γ recombinant proteins were subjected to SDS–PAGE and transferred to PVDF membrane (Millipore, Milan, Italy). The bovine serum albumin (BSA) was used as control of unspecific binding, equal loading of BSA and recombinant YB1 or p63 proteins in Far-Western blotting was verified by coomassie staining (data not shown).

Luciferase reporter assay

MDA-MB-231 cells were cotransfected with ΔNp63α, PIK3CA luc-promoter and pRL-TK. YB-1 gene silencing was carried out 24 hrs before plasmid transfection. At 24h after transfection, cells were harvested in 1x PLB buffer (Promega) and luciferase activity was measured using Dual Luciferase Reporter system (Promega) using pRL-TK activity as internal control. FireFly-derived luciferase activity was normalized for transfection

efficiency. Successful transfection of p63 and silencing of YB-1 was confirmed by immunoblotting.

RNA Immunoprecipitation

1 x 106 MDA-MB-231 cells were seeded in 100mm plates and transfected with pcDNA3.1 or ΔNp63α espression vector. 24h after transfection, cells were fixed with 1% formaldehyde for 10 min and washed twice in ice-cold PBS. Cell extracts were prepared in RNA-immunoprecipitation buffer (0.1% SDS, 1% Triton, 1mM EDTA, 10mM Tris pH 7.5, 0.5mM EGTA, 150mM NaCL) supplemented with complete protease inhibitor mixture (Sigma Chemical Co., St. Louis, MO, USA) and sonication was carried out with BANDELINE SONOPULSE HD2200 instrument under following conditions: 8 pulses of 4 sec at 0.250% of intensity. Cell extracts were incubated with anti-YB-1 (3µg) at 4°C overnight. The RNA-protein immunocomplexes were precipitated with protein A beads (Roche) satured with tRNA and, after reverse cross-link, subjected to RealTime PCR. For PCR analysis total RNA was isolated using the RNA Mini Extraction Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 µg) was used to generate reverse transcribed cDNA using SuperScript III (In Vitrogen Life technologies, Inc.). PCR analysis was performed with the following primers:
Twist (F): 5' AGAAGTCTGCGGGCTGTG
Twist (R): 5' TCTGCAGCTCCTCGTAAGACT
YB-1 (F): 5' CGCAGTGTAGGAGATGGAGAG
YB-1 (R): 5' GAACACCACCAGGACCTGTAA
HPRT (F) : 5' CCT GCT GGA TTA CAT TAA AGC
HPRT (R) : 5' CTT CGT GGG GTC CTT TTC
The amplification sequence consisted of 30 cycles of 94°C/1', 55°C/1', 72°C/1'. PCR products were resolved by 2% agarose electrophoresis. RT-PCR amplification results were analyzed by Quantity One software (Biorad). Real Time PCR was performed with a 7500 RT-PCR Thermo Cycler (Applied Biosystem) as already described (Di Costanzo et al., 2010). HPRT was used for normalization. The results were expressed with the value relative to HPRT (set at 1) for each mRNA sample. For Snail1 amplification we used Quantitect Primer Assay/Hs Snail1 from Quiagen.

Immunofluorescence

H1299, MDA-MB-231 or MCF7 cells (5.0 ×105) were plated in 35 mm dish, grown on micro cover glasses (BDH) and transfected with 0.2 µg of pcDNAYB-1/GFP plasmid with or

without 0.6 μg of ΔNp63α or ΔNp63γ plasmid. The GFP vector transfected with ΔNp63α or ΔNp63γ encoding plasmids was used as control (data not shown). At 24 hrs after transfection, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich, Germany) for 15 min at 4°C. Cells were permeabilized with ice-cold 0.1% Triton X-100 for 10 min and then washed with PBS. P63 was detected by using a 1:200 dilution of the monoclonal antibody 4A4. After extensive washing in PBS, the samples were incubated with Cy3-conjugated anti-mouse IgGs at room temperature for 30 min. After PBS washing, the cells were incubated with DAPI (4′,6′-diamidino-2-phenylindole; 10 mg/ml [Sigma-Aldrich, Germany]) for 3 min. The glasses were mounted with Moviol (Sigma-Aldrich, Germany) and examined under a fluorescence microscope (Nikon). Images were digitally acquired and processed using Adobe Photoshop software CS3.

Immunohistochemistry

Human corneal tissues obtained from the Veneto Eye Bank Foundation (Zelarino-Venezia, Italy) were collected at different times, from 5 to 120 hours after death. The corneas were evaluated and divided into two groups: (1) normal corneas (typically fresh tissues), and (2) wounded corneas, as previously described by Di Iorio et al., 2005. The corneas were managed in accordance with the guidelines in the Declaration of Helsinki for research involving human tissue. They were fixed in 3% paraformaldehyde (overnight at 4°C), embedded in optimal cutting temperature [OCT] compound, frozen, and sectioned (5- to 7−μm-thick slices). Sections were immunostained using antibodies against pan-p63 (4A4, mouse monoclonal, 1:100, BD Biosciences, Milan, Italy), keratin 12 (K12, sc-17099, goat polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and YB-1 (Cell Signaling Technology, Beverly, Massachussets, USA). Rhodamine and fluorescein isothiocyanate-conjugated secondary antibodies (1:100; Santa Cruz Biotechnology) were incubated for 1 h at room temperature. Specimens were analyzed with an LSM 510 Meta Confocal Microscope (Zeiss SpA, Milan, Italy).

Time-lapse microscopy

H1299 cells were cultured on 35 mm dishes (Corning, NY) at a density of 2×104 cells/dish. ΔNp63α expression was induced with 2 μg/ml doxycycline for 48 hr. 16 hr after transfection, cell dishes were placed in a mini-incubator connected to an automated stage of an optical microscope (Cell^R, Olympus

Co., Japan). Time zero images of selected position were collected in fluorescence in order to localize transfected cells. Then, at the same positions, images were recorded in brightfield (BF) every 10 minutes for 12 hours. BF images were mounted to obtain 72 frames time lapse video per each position. Cell trajectories were reconstructed from time lapse videos using Metamorph software (Molecular Device, CA). Root mean square speed (S) and persistence time (P), were chosen as relevant parameter in order to describe the macroscopic features of cell migration of the different experimental conditions. These parameters were calculated according to the procedure reported by Dunn (Dunn G.A, 1983). Briefly, mean-squared displacement (MSD) of each cell, is calculated according to overlapping time intervals method (Dickinson RB and Tranquillo RT, 1993). Subsequently, S and P are estimated by fitting the experimental data of the MSD to a linear approximation of the persistent random walk model. The upper limit for the data fitting was set approximately at 200 min per each cell owing to the deviation from linearity that is observed at higher time points. The fitting procedure provided (S, P) pairs per each cells and Statistical significance between S and P values of the different experimental setups was assessed by performing a non-parametric Kruskal-Wallis test in Matlab (MathWorks, MA). p values < 0.05 were considered significant.

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