トロフィー，リソソーム病のほか，ちょっと聞き慣れないかもしれませんが，異染性脳白質異栄養症，ニューロゴーシェ，ムコ多糖症，ハンター症候群，ハーラー症候群などの子供たちの酵素補充の供給源として使うことができます。

小室　いろいろな供給源から取ってきた間葉系の細胞は同じものなのでしょうか。

梅澤　形態学的にはとてもよく似ており，すべて線維芽細胞のように見えますが，実際はまったく違います。増殖期における細胞の遺伝子発現をDNAチップで網羅的に調べると，取ってきた元の組織の形質を見事に維持しているのです。たとえば子宮内膜に由来するものは，ホルモンの受容体の発現が高く，骨髄由来の細胞は骨形成因子であるBMPの発現が高い。軟骨の場合はDNAチップだけではなく，ランダムシークエンスによる解析を行っても，遺伝子発現のトップ10は基質に由来するものばかりです。

しかし，間葉系の細胞は*in vitro*では多分化能をもっています。たとえば脂肪細胞由来であっても脂肪のほかに骨，骨格筋に分化させることができます。ただ，増殖期においては元の組織の形質を保持しているのです。

間葉系の細胞が元の組織の形質を保持しているのは，おそらくメチル化で規定されているためだと考えられます。つまり，ゲノム上にきちっとしたエピジェネティクスが刷り込まれていて，たとえ分裂しても同じメチル化が維持されるため，決してほかの細胞になることはありえない。ですから元の組織の枠には入っているけれども，その枠の中では多分化能をもっているのです。

■間葉系幹細胞の起源と治療におけるメリット

小室　いろいろなところから間葉系の細胞が取れるというお話でしたが，その中に間葉系の幹細胞がごく一部入っているという理解でいいのですか。

梅澤　これは二つの可能性があります。幹細胞がごく一部存在しているのだとしたら，混ざった状態で培養すると分化が進んだ前駆細胞は増殖できませんので，最終的には増殖能をもつ間葉系幹細胞が集団のほとんどを占めるようになると考えられます。

もう一つは，試験管の中ではある程度コミットした細胞が脱分化し，多分化能を有しているだけで，結局どの細胞も一緒で，間葉系幹細胞という細胞自体はまったく存在しないという説もあります。

桜田　起源細胞についての研究はまだあまり進んでいないのです。非造血系の幹細胞を組織から分離し，そのままFACSで同定するという研究は非常に遅れています。これは，たとえば骨髄中では血液系の細胞と比較して非造血系の比率が非常に小さいからです。その起源になる細胞はいったい何なのかというのは間葉系の幹細胞の今後の研究でもとくに重要なポイントだと思います。

梅澤　そうですね。間葉系のよい点は，大量に増やすことができることです。骨髄由来の間葉系幹細胞は1回成功すれば30回は分裂しますので，1つの細胞から10^9（$=2^{30}$）以上の細胞を得ることができます。

小室　ヒト由来の骨髄幹細胞でも可能なのですか。

梅澤　ヒトでもまったく問題なく増えます。ただし，最初の分裂が起こるかどうかがかなり重要です。たとえば臍帯血由来の間葉系は，4検体に1検体ぐらいしか増えてこない。一方，月経血などは100%増えてきます。骨髄由来の間葉系細胞に関しては，産業技術総合研究所の培養技術は非常に進んでいて，患者の血清濃度15%の条件下で失敗なく増やすことができています。臨床においては培養した細胞が100%分裂できることが要求されます。

従来，骨の欠損に対しては，腸骨を取り出して砕いたものを欠損部に入れる方法がとられてきました。腸骨を採取することは非常に痛みを伴います。ですから骨髄由来間葉系細胞が培養できて増やすことができるのであれば，大変大きなメリットになります。にもかかわらず，この治療が遅れているのは，間葉系幹細胞の骨への分化能が完璧ではないからです。骨のマーカーはあるけれども見事な骨，硬い骨ができるかというとなかなか難しい。

これは軟骨においても同様です。たとえば，お相撲さんの軟骨が壊れているので新たにつくって入れてあげる場合，軟骨は弾性が重要ですが，荷重に耐えられるような軟骨ができない現状なので間葉系幹細胞ではなく，ほとんど増えない軟骨細胞が使われているのです。

さらに形成外科の領域で間葉系を移植すると，傷の治りが非常にいいという説も知られています。

小室　それはどうして治りがよいのですか。血管ができるからではなくて，皮膚ができるからですか。

梅澤　皮膚の場合，治りがよいというのは瘢痕ができないということです。周りの膠原線維の産生が低下すると見た目がきれいに治癒します。これは，移植した骨髄の間葉系細胞自体がきれいな真皮をつくるという説と，間葉系が出しているサイトカインが傷の治りをよくするという説があります。間葉系細胞を打たなくても，bFGF といったサイトカインを打てば傷がきれいに治るのは研究レベルで知られています。

■どこまで臨床応用されているか

小室　間葉系幹細胞は ES 細胞のように何にでも分化するのではなく，部分全能性しかありません。いまのところ，分化するとわかっている細胞にはどういうものがありますか。

梅澤　骨，軟骨，脂肪，骨格筋，心臓，神経です。肝臓については，αフェトプロテインは出るけれども，アルブミンは出てこないという理由から，肝様細胞とされています。また，膵β細胞は ES 細胞と同じ問題点がありまして，インスリンを産生するものの，グルコースに反応せず，神経のマーカーはいつも同時に出ているというのが現状です。

小室　そのようにかなり多くの細胞に分化することはわかっているけれども，骨も軟骨も完全ではないわけですね。臨床に使われている例ではどんなものがありますか。

梅澤　米国ではリソソーム病の治療に，酵素の補充を目的に間葉系細胞が使われています。この場合は他家移植ですので，ユニバーサル ES に対してユニバーサルな間葉系幹細胞といわれています。異染性白質ジストロフィーやムコ多糖症 VII 型の患者にも，酵素補充を目的に，ユニバーサルな間葉系幹細胞が使われています。

小室　その幹細胞はどこから取ってきたのですか。

梅澤　骨髄です。骨髄由来の幹細胞は，患者が必要とする酵素もレベルは非常に低いけれども出しています。現在は酵素補充療法でやっていますが，もともとの細胞が生着するだけで酵素を産生してくれるので助けることが可能です。ユニバーサル細胞にするために特別に行うことはありませんが，品質管理規則（GMP）の基準を満たす必要はあります。

小室　米国 Osiris Therapeutics 社が樹立した細胞はユニバーサルではないのですか。Osiris 社の間葉系幹細胞は，アロ抗原による拒絶反応は問題にならないのですか。

梅澤　Osiris 社の細胞はユニバーサルな間葉系細胞です。また，ご指摘通り，アロ抗原による拒絶反応は問題になります。間葉系の細胞表面に HLA-DR は出ていませんが，クラス I 抗原が出ていますので，私は拒絶されると思います。しかし Osiris 社の発表では「拒絶されにくい」

ということにはなっています。免疫抑制薬は必要になります。

リスクとベネフィットのバランスを考えてみたいと思います。たとえば先天性代謝疾患症の子供たちは致死的で，日本では代替治療がありません。免疫抑制薬が必要だとしても，間葉系幹細胞による酵素補充療法を行うことができるのであれば，これはきわめて重要な第一歩になる。

また，筋ジストロフィーも非常に重篤な疾患です。もし，細胞治療が筋ジストロフィーに有効であるならば，最終的には全身の筋肉に対して細胞が必要です。ですから，無尽蔵に得られるES細胞や間葉系幹細胞による治療は大きなベネフィットだと思います。

ここで重要なのは，移植した細胞そのものが筋肉になる必要はないことです。ES細胞が骨格筋と融合し，ES細胞側の正常なジストロフィー遺伝子が発現するようになればジストロフィン蛋白の補充になります。融合さえしてくれればよいのです。

■細胞治療の発展していく方向

小室　もうすでにOsiris社からユニバーサルな間葉系細胞が売られていますが，梅澤先生としては間葉系による細胞治療はどの方向に進むと思いますか。ユニバーサルなものが主流になるのか，それとも先生が最初に言われたようにいろいろなところから間葉系が取れるので，本人，家族のものを使う方向にいくのか。どちらでしょうか。

梅澤　ユニバーサルな間葉系幹細胞は，規格化されていることにメリットがあって，後々の臨床でも同じ基準で行うことができます。つまり，細胞を化合物の薬と同じように使うことができるのです。一方，各個人の細胞を取って増やすオーダーメイド医療は，細胞をGMP基準のセルプロセッシングセンターで増やして体内に戻すことになりますが，これはなかなか普及しにくいのではないかと予想しています。

小室　Osiris社の細胞はどんどん使われているのですか。

桜田　臨床試験の最終段階にきていますが，実際には市場に出るのはもう少し先になりそうですね。

小室　2，3年前には骨にかなり移植していて，2年前のアメリカ心臓学会（AHA）では心臓に応用すると，Osiris社の社長自身が言っていましたね。

桜田　今後FDAの基準に従った臨床試験をクリアしていかないと最終的には難しいと思います。

小室　拒絶反応はないと断言していましたが，本当ですかね。

桜田　先日ボストンで開催されたISSCR（International Society for Stem Cell Research）では，プロスタグランジンや，免疫抑制のサイトカインが間葉系幹細胞から産生されるためだろうといわれていました。ただ，分化した後，その状態が維持できるかどうかについては，今後の課題だと思います。

体性幹細胞研究と応用

■心臓疾患での現状

小室　心臓では骨格筋芽細胞を心臓に移植するという試みが実際に行われています。骨格筋では，分化しきっていないサテライト細胞が存在しているので，患者の大腿の筋肉を10～20g取ってきて培養すれば，骨格筋芽細胞を増やすことができます。これを患者の心臓に植えて，同じ横紋筋としての作用を期待するという治療法です。欧米数ヵ国で10例以上行われていますが，一応は心臓の機能がよくなったという結

果が出ています。ただ，骨格筋芽細胞は心臓という環境においても骨格筋にしか分化しないので，不整脈という問題があり，ほとんどの人が抗不整脈薬を飲まなければなりません。または心室細動が起こるので除細動器を植え込まざるをえないという状態です。ですからやはり心筋に分化する細胞が望まれているのです。

われわれを含めて世界で3ヵ所が，心臓から一種の幹細胞様の細胞をSP細胞やSca-1というマーカーを使って取ることに成功しています。これらの細胞をある種の条件下で培養すると，心筋に分化し，体に戻すといろいろな細胞に分化することを確認しました。しかし，心臓から細胞を取って心臓に植えるのは臨床的にどうかと思い，われわれは移植というより，生体内の心筋幹細胞の性質を明らかにして生体内で治す方向で研究しています。

■神経研究の現状と問題点

小室　心臓に比べてもっと進んでいると思われる神経のほうはいかがでしょうか。

桜田　臨床的なニーズは病気によってすごく違いがありますが，神経の再生医療では，最終的には脳の機能を回復させることが目的です。この場合は単に神経，あるいは神経系の細胞ができただけでは不十分で，回路を再構築しないといけないという大きな問題があります。しかし神経回路と脳の機能の関係はまだ完全には明らかにされていないのです。

また，脳がもっている再生の力を理解し，それが不足するところを治療によって補おうという戦略は非常に重要視されています。実際，成人の脳の中にある神経幹細胞自体もある程度再生する力があることは，少なくともげっ歯類では報告をされていて，実際に障害を治す力もあることも報告されています。それではなぜ病気は治らないのかといいますと，次の三つの説があげられています。一つは，元 東京大学の中福先生（現 オハイオ大）が実証されていることですが，非常に大きな障害の場合には，内的な力だけでは治しきれない。しかしこの場合，サイトカインを補充することで内的再生力を高めることにより治すことができる可能性はあります。二つ目は，神経変性疾患では，神経の再生能を抑えてしまうという説です。アルツハイマーの場合はβアミロイドの蓄積が，パーキンソン病の場合はドパミンの欠乏が脳内の神経再生を非常に顕著に抑制することが知られています。ですから，単に細胞治療を行うだけではなく，再生を抑制する部分を解除する必要があります。三つ目は幹細胞自体が老化すると，その機能が徐々に落ちてくるという説です。この場合は細胞治療が必須となると考えられます。

臨床上用いられている薬剤による内的な神経再生の促進という観点では，抗うつ薬，スタチン系の薬剤，それからPDE-5の阻害薬（バイアグラ®）にニューロン新生促進活性があることが注目されています。なかでも抗うつ薬に関しては神経再生促進活性が薬効と直接関係することが2003年の夏に報告されました。この報告は，言い換えるとヒトの成人でも神経再生活性をある程度保持していることを示していて，今後新しい薬剤の開発により，内科的な神経再生のアプローチが可能になるのではと考えています。

小室　抗うつ薬による神経再生促進はとてもおもしろいと思いますが，どのようなメカニズムなのですか。

桜田　抗うつ薬はセロトニン作動性あるいはアドレナリン作動性の神経を活性化させる効果があり，これらの神経が活性化しますと現時点では仮説ですが，BDNFやFGFといった神経再生活性のあるサイトカインが海馬の近くに誘導され，神経再生を誘導するのではないかといわれています。ですから，間接的に神経幹細胞の再生を促進していると考えられています。

小室　バイアグラとスタチンはどうですか。

桜田　バイアグラはPDE-5阻害薬なので細胞内情報伝達系でNOの産生の促進などが背景になっていると考えられます。スタチン系についてはVEGF（血管新生促進因子）の産生を促進することで神経再生や増殖，分化を促進している可能性が考えられています。

■神経再生に向けた臨床試験

桜田　死亡胎児の脳を移植する治療はすでに臨床応用されています。スウェーデンを中心に実施された非盲検試験では多数の有効例が報告されましたが，最近米国で実施された二つの二重盲験試験では無効との報告がなされました。もちろん本試験の結果は中枢神経系の細胞治療のコンセプトを否定するものではなく，米国を中心にさまざまな細胞治療の開発が進められています。たとえば，マイクロビーズに培養したヒトの網膜色素上皮細胞を付着させてパーキンソン病患者に移植する試験や，培養神経幹細胞や間葉系幹細胞を神経再生に応用しようという研究・開発が実施されています。

梅澤　死亡胎児の脳を移植した臨床試験では，免疫抑制薬を使っているのでしょうか。

桜田　従来スウェーデンなどで行われた非盲検試験では免疫抑制薬を使っていましたが，アメリカの二重盲検試験では免疫抑制薬を使いませんでした。しかし，生着率が非常に悪いわけではないので，効かなかった原因として，細胞の処理法や移植の方法が議論されています。ただ，去年（2003年）報告されたアメリカの臨床試験では，最初の6ヵ月は非常に効いています。しかし2年たつと効果がなくなっているので，今後その原因を解明する必要があると思います。

ただ，これはあくまで死亡胎児の脳を入れる方法で，ES細胞や神経幹細胞を用いた試験とは明らかに違いますから，これをES細胞とか神経幹細胞を用いた治療と同じように考える必要はまったくなく，あくまで臓器移植のようなかたちでの移植の結果だと解釈しています。

■細胞治療が有効な脳疾患

小室　脳の細胞移植としては，対象疾患は現在パーキンソン病だけなのですか。

桜田　多発性硬化症に神経幹細胞が有効だというのは去年報告されていますし，ハンチントン舞踏病においても有効だという報告もあります。脊髄損傷における有効性も慶応大学の岡野先生のグループから報告されています。

小室　その場合は移植するのですか。

桜田　そうですね，移植します。多発性硬化症は脳全体に広がる疾患ですが，成人の神経幹細胞の末梢血投与が有効であることが，昨年報告されました。多発性硬化症の場合は脱髄といって神経回路とは違う面がありますので，移植によって回路が形成されたとはいえません。しかも自己免疫疾患的な特徴がありますので，やはり疾患によって違いがあると思います。

ただ，ラットとヒトの脳は明らかに違うでしょうから，将来どうやって動物試験の結果を外挿していくかが問題です。サルの試験などで，前臨床試験を行う必要もありますね。

小室　心臓の場合は，心筋梗塞で心筋がなくなったところに心筋を植えるという単純な発想です。しかし脳では神経回路をつくるところに問題があるのですね。

桜田　それに関してもやはり多様で，それぞれの疾患別に最適な方法は違うと思います。パーキンソン病のように，回路のいらない，ドパミン補充を目的とした治療もありますので。

梅澤　Verfaillie博士（ミネソタ大学医学部幹細胞研究所）が成体万能幹細胞（MAPC）と呼ばれる，骨髄に存在する非血球系の体性幹細胞を脳梗塞治療に使われたそうですが，これは有効なのでしょうか。

桜田　動物試験のデータではMAPCの移植が

脳梗塞に効くことが示されています。また，間葉系幹細胞の移植が効くという報告もさまざまなグループから出ています。脳梗塞は，ある程度機能を回復する例が報告されている疾患ですので，脳内における神経幹細胞による再生だけではなく，骨髄や末梢血由来の細胞による再生の仕組みが生理的に存在している可能性があります。このような末梢由来細胞による治療効果は，神経に分化して保護しているのか，再生因子分泌というパラクライン作用なのかは今後の研究課題だと思います。少なくともGFPで標識した間葉系細胞が大量に脳内に入るというのは確かなようです。

小室　心筋梗塞の場合，骨髄を移植しても心筋になる率は非常に低いといわれていますが，脳梗塞の場合は骨髄が神経細胞になるということはあるのですか。

桜田　神経細胞になるという報告はたくさんあるのですが，このようにしてできた神経細胞が長期間維持されるのかどうかは不明です。末梢血由来の細胞が組織修復に働くのかどうかは，150年前ドイツのJulius Cohenheimが提案して以来，喧々諤々で今も結論は出ていません。心筋梗塞についても骨髄は心筋にならないとされていますが，なるという報告をされている方もいます。骨髄からの心筋分化を報告されている研究でも，造血系からの分化は否定されています。移植のタイミングや炎症の状態によって分化の効率が変わるのかもしれません。また，すべての病態で同じように骨髄が寄与していると解釈する必要はないと思います。

■細胞移植のいちばんの成功例は虚血下肢例

梅澤　いま細胞治療で注目を浴びているのは，虚血下肢における骨髄と末梢血の移植であると理解しております。心臓に対して骨髄を移植しても骨髄細胞が心筋にならないとしたら，血管になるのかもしれないし，あるいはただ単に誘導しているだけかもしれないですね。

小室　おっしゃるように細胞移植でいちばん進んでいて，有効性が最も認められているのは下肢虚血に対してといえますね。いまは骨髄の単核球を移植する方法がよいとされています。われわれは末梢血の単核球を移植しているだけですが，それもかなり有効で，われわれの成績でも7，8割の方が足の切断を免れています。下肢虚血は血管を増やせばいいわけですが，心臓に関しても虚血性の心疾患は血管を増やせばいいので，同じ手法が使えるのではないかと思います。

あと，骨髄を打つことによって心筋ができるかどうかに関しては，私はかなり否定的です。しかし，血管ができることで，間接的に再生が促進される可能性はあると考えています。

山下　心臓の機能回復に関しては，心筋そのものができればいちばんいいのでしょうが，心筋ができなくても血管ができれば機能は回復することもありますし，さらには線維芽細胞ができればカチカチの結合織の弾性を回復することもあると思います。心筋が再生できて動くようになるのが理想的ですが，治療効果としては心機能の回復が得られ，それが安全かつ簡単で効果的であれば治療としては非常に有効です。治療の効果があればいいのであって，メカニズムがどれであっても問題ないと考えています。

小室　そうですね。ただ，メカニズムを誤解していると進歩がなかったり，違う方向に進んだり，また違ったものをつくったりということがあるので，やはり正しい理解は必要だろうと思います。

山下　そうですね。どういうメカニズムで治っているかというのは理解しないといけませんが，よくなった理由がはっきりわかれば心筋ができていなくてもよいと思います。

桜田　確かに必ずしも心筋を100%すべてに目的としなくてもいいかもしれない。ただ，心筋

の再生が必要な疾患も必ずあると思います。

小室　ES細胞，間葉系の幹細胞，また体性幹細胞の現状と課題についてもよく理解できました。これから研究が進歩して，実際に臨床に使われる日も近いと思いますので，皆さんの研究の発展を期待しています。どうもありがとうございました。

Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex

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The critical immortalizing activity of the human papillomavirus (HPV) type-16 E6 oncoprotein is to induce expression of *hTERT*, the catalytic and rate-limiting subunit of telomerase. Additionally, E6 binds to a cellular protein called E6-associated protein (E6-AP) to form an E3 ubiquitin ligase that targets p53 for proteasome-dependent degradation. Although telomerase induction and p53 degradation are separable and distinct functions of E6, binding of E6 to E6-AP strongly correlated with the induction of *hTERT*. Here, we demonstrate using shRNAs to reduce *E6-AP* expression that E6-AP is required for E6-mediated telomerase induction. A yeast two-hybrid screen to find new targets of the E6/E6-AP E3 ubiquitin ligase complex identified NFX1. Two isoforms of NFX1 were found: NFX1-123, which coactivated with c-Myc at the *hTERT* promoter, and NFX1-91, which repressed the *hTERT* promoter. NFX1-91 was highly ubiquitinated and destabilized in epithelial cells expressing E6. Furthermore, knockdown of NFX1-91 by shRNA resulted in derepression of the endogenous *hTERT* promoter and elevated levels of telomerase activity. We propose that the induction of telomerase by the HPV-16 E6/E6-AP complex involves targeting of NFX1-91, a newly identified repressor of telomerase, for ubiquitination and degradation.

[*Keywords*: Telomerase; HPV; transcriptional repressor; ubiquitin; E6; E6-AP]

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Immortalization is a critical step in the process of transformation (Hahn et al. 1999a; Elenbaas et al. 2001). Several recent investigations have determined that the combined effects of disruption of the Rb/p16 pathway and induction of telomerase activity are sufficient to immortalize human epithelial cells (Kiyono et al. 1998; Garbe et al. 1999; Lundberg et al. 2002). The human papillomavirus (HPV) type-16 oncoproteins, E6 and E7, target these pathways to efficiently immortalize primary epithelial cells (Kiyono et al. 1998; Fehrmann and Laimins 2003). Although the E7 oncoprotein abrogates the Rb/p16 pathway by disrupting the Rb/E2F interaction and targeting Rb for degradation (Boyer et al. 1996; Helt and Galloway 2001), the E6 oncoprotein promotes the degradation of p53 through its interaction with the cellular E6-associated protein, E6-AP, an E3 ubiquitin ligase (Scheffner et al. 1993). In addition, E6 induces telomerase activity, thereby contributing to the immortalization of epithelial cells by maintaining telomere length (Klingelhutz et al. 1996). Studies of mutated E6 proteins reveal that induction of telomerase is an independent function of E6 separable from p53 degradation and that telomerase activation rather than p53 degradation is the critical step for immortalization of epithelial cells (Klingelhutz et al. 1996; Kiyono et al. 1998).

Several groups, including ours, have found that activation of telomerase by E6 is due to its ability to induce expression of *hTERT*, the catalytic and rate-limiting subunit of telomerase (Gewin and Galloway 2001; Oh et al. 2001; Veldman et al. 2001). Given that the majority of tumor cells have induced expression of *hTERT*, the mechanisms regulating the *hTERT* promoter are the subject of extensive investigation. Although the *hTERT* promoter region lies within a CpG island, promoter methylation does not appear to be the primary mode of silencing *hTERT* expression in telomerase negative cells (Devereux et al. 1999; Dessain et al. 2000). Chromatin conformation, on the other hand, does seem to play a key role in *hTERT* activity. Several studies have demonstrated that inhibition of histone deacetylases by trichostatin A in certain telomerase-negative cells can alter the

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chromatin structure and induce telomerase expression (Cong and Bacchetti 2000; Takakura et al. 2001; Hou et al. 2002). Interestingly, induction of telomerase by E6 also seems to involve an epigenetic mechanism that regulates the extent of *hTERT* induction. Different clones of human foreskin keratinocytes (HFKs) transduced with E6 express varying levels of *hTERT* that increase as cells are passaged in culture despite no accompanying increase in E6 expression levels (Klingelhutz et al. 1996; Kiyono et al. 1998; Baege et al. 2002).

The *hTERT* promoter contains many known transcription factor-binding sites, including several E boxes and Sp1 sites (Horikawa et al. 1999; Takakura et al. 1999; Wick et al. 1999). Although c-Myc is able to bind the E boxes in the *hTERT* promoter and can induce *hTERT* expression in many cell types (Wang et al. 1998; Greenberg et al. 1999; Wu et al. 1999), we and others have found no correlation between c-Myc expression and the ability of E6 to induce *hTERT* (Gewin and Galloway 2001; Oh et al. 2001; Veldman et al. 2001). We have, however, determined that the promoter region including the proximal E box is important for E6-mediated *hTERT* induction (Gewin and Galloway 2001; Veldman et al. 2001).

In an effort to elucidate the mechanism by which E6 induces *hTERT*, we began to characterize the ability of E6 and mutated versions of E6 to induce *hTERT*, to target p53 for degradation, and to bind to E6-AP. A strong correlation between E6-AP binding and *hTERT* induction prompted the search for possible new targets of the E6/E6-AP complex by a yeast two-hybrid screen. In addition to several known targets of E6/E6-AP, the screen identified a transcriptional repressor known as NFX1. This work describes both a requirement for E6-AP and a role for NFX1 in E6-mediated *hTERT* induction.

Results

E6-AP is required for telomerase activation of HPV-16 E6

The initial discovery that E6 induces expression of hTERT immediately prompted speculation that this function might be linked to the well-established p53 degradation function of E6. Although overexpression of p53 has been shown to repress *hTERT* expression in some tumor cell lines (Kanaya et al. 2000; Xu et al. 2000), inactivation of p53 is insufficient to induce telomerase (Hahn et al. 1999a; Opitz et al. 2001). Furthermore, *hTERT* induction and p53 degradation are separable and distinct functions of E6 as indicated by E6 mutants such as F2V, 8S/9A/10T, and Y54H that can activate telomerase but do not target p53 for degradation (Table 1; Klingelhutz et al. 1996; Liu et al. 1999). Yet, review of several published studies characterizing mutants of E6 revealed that mutants that retain the ability to target p53 for degradation could also induce *hTERT* expression (Table 1; Foster et al. 1994; Dalal et al. 1996; Zimmermann et al. 1999). As the E6/E6-AP E3 ubiquitin ligase complex targets p53 for degradation, binding of E6 to E6-AP may also be required for *hTERT* induction. Additional in vitro E6/E6-AP binding studies (data not shown), as well as published data (Table 1), revealed a strong correlation between E6 binding to E6-AP and telomerase activation. All of the mutated E6 proteins that could bind E6-AP induced *hTERT* expression or immortalized post-M0-mammary epithelial cells.

To further support the hypothesis that E6-AP binding is required for E6 to induce *hTERT* expression, we developed short-hairpin RNA (shRNA) retroviral constructs to reduce *E6-AP* expression in primary HFKs. These experiments were performed using constructs to stably express the shRNAs driven by an RNA polymerase III promoter, either U6 or H1 (Paddison et al. 2002; Grandori et al. 2003; Smith et al. 2003). Three different 26- to 29-nt regions of the *E6-AP* gene were targeted to identify a construct that would give the greatest decrease in *E6-AP* expression. Figure 1A shows shRNA1 (esh1) was the most effective of the three, reducing expression of *E6-AP* RNA to ~29% of that in the vector-control infected cells. The other two hairpins (esh2 and esh3) exhibited intermediate levels of effectiveness.

HFK cell lines stably expressing the E6-AP shRNAs were subsequently infected with empty vector or E6 retroviral constructs. Figure 1B demonstrates that knock-

Table 1. *Telomerase activation and E6-AP binding of E6 proteins*

HPV 16E6 protein	TRAP activity	E6-AP binding	p53 degradation	References
16E6 wt	+	+	+	Klingelhutz et al. 1996; Liu et al. 1999
16E6 F2V	+	+	-	Liu et al. 1999; data not shown
16E6 8S/9A/10T	+	+	-	Foster et al. 1994; Klingelhutz et al. 1996; Gewin and Galloway 2001
16E6 Δ9–13	-	-	-	Foster et al. 1994; Gewin and Galloway 2001
16E6 L50G	-	-	-	Zimmermann et al. 1999; data not shown
16E6 Y54H	+[a]	+	-	Liu et al. 1999
16E6 Δ118–122[b]	-/+[a]	-/+	-/+	Foster et al. 1994; Dalal et al. 1996; Kiyono et al. 1998
16E6 Δ123–127	-	-	-	Foster et al. 1994; Klingelhutz et al. 1996; Liu et al. 1999
16E6 Δ146–151	+	NT	+	Foster et al. 1994; Klingelhutz et al. 1996; Kiyono et al. 1998

(NT) Not tested.
[a]TRAP activity is inferred by the ability of these mutants to immmortalize mammary epithelial cells (MECs).
[b]16E6 Δ118–122 appears to have low-level activity in all of these functions.

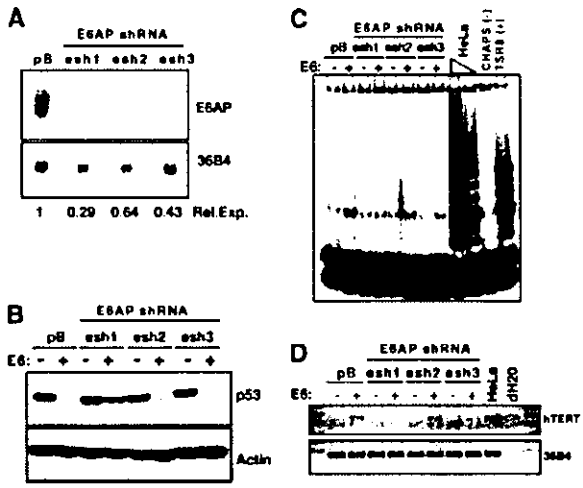

Figure 1. *E6-AP* expression is required for telomerase induction by HPV 16E6. (*A*) Northern blot. Three shRNA constructs targeting *E6-AP* (esh1,2,3) were transduced into HFKs and RNA was harvested to examine levels of *E6-AP* message. *36B4* is a loading control. Relative expression levels are presented normalized to *36B4* and the pB empty vector control. (*B*) Western blot. HFKs expressing the *E6-AP* shRNA constructs were subsequently transduced with LXSN empty vector (−) or LXSN-16E6 (+). p53 levels were examined by Western blot. Actin is a loading control. (*C*) TRAP assay. Extracts from the same cells shown in *B* were assayed for telomerase activity. HeLa cells are a positive control lysate. CHAPs is a lysis buffer negative control. TSR8 is a synthetic template of eight telomeric repeats used as a PCR positive control. (*D*) RT–PCR. Expression of *hTERT* RNA was examined by RT–PCR of RNA extracts. *36B4* is a loading control.

down of *E6-AP* abrogated the ability of E6 to target p53 for degradation, with esh1 again proving to be the most effective of the three shRNA-expressing cell lines. In addition, assays for telomerase activation revealed that *E6-AP* depletion disrupted E6-mediated *hTERT* induction in HFKs (Fig. 1C). TRAP activity in these cells directly correlated with the level of *hTERT* RNA detected by RT–PCR (Fig. 1D). These experiments demonstrated that *E6-AP* expression was required for *hTERT* induction by HPV-16 E6.

A new target of E6/E6-AP

Our studies revealed that E6 must bind to E6-AP but not necessarily to p53 in order to induce *hTERT*. A survey of previously identified cellular targets of E6/E6-AP did not reveal any logical contenders for *hTERT* transcriptional regulators; therefore, we began to search for new targets of E6/E6-AP by a yeast two-hybrid screen. The bait construct consisted of a catalytically defective E6-AP (C833A) fused to the Gal4 DNA-binding domain. To ensure equal dosage of both E6 and E6-AP, the plasmid also encoded the E6 oncoprotein. In a screen of both fetal brain and HeLa cell cDNA libraries, several known E6/E6-AP interactors were identified, including p53, HHR23A (human homolog of *rad23*), and a homolog of the *E6TP1* gene (Unigene Hs. 406879; Scheffner et al. 1993; Gao et al. 1999; Kumar et al. 1999). In addition, a new E6/E6-AP target protein identified in the screen was NFX1 (nuclear factor binds to the X1 box), a transcriptional repressor of MHC class II genes (Song et al. 1994). NFX1 was originally described and cloned in a screen for proteins that bound to the X-box region of MHC class II genes and is thought to be involved in a feedback loop to limit the immune response following infection (Song et al. 1994).

Recent advancements in the annotation of the human genome revealed that *NFX1* has two splice variants encoding isoforms with identical N termini and variant C termini (Fig. 2A, Unigene Hs. 413074). We will specify the longer 1120-amino acid isoform here as NFX1-123, as it is ~123 kDa, and the shorter 833-amino acid isoform as NFX1-91 (~91 kDa). Both isoforms have a RING finger/PHD finger domain; found in many E3 ubiquitin ligases, this domain has been shown to confer autoubiquitination activity to NFX1 in in vitro assays (Lorick et al. 1999). The RING finger domain is followed by several cysteine/histidine-rich sequences identified as NFX1-type zinc fingers by the Pfam database (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01422). Identified as the DNA-binding domain of NFX1 (Song et al. 1994), these zinc fingers [C-X(1–6)-H-X-C-X3-C-(H/C)-X(3–4)-(H/C)-X(1–10)-C] are highly homologous to one another but do not exhibit the typical spacing of traditional zinc fingers. NFX1-91 contains the first six zinc fingers followed by a unique lysine-rich stretch of 25 amino acids, whereas NFX1-123 contains eight zinc fingers as well as a region known as an R3H domain believed to be involved in single-stranded nucleic acid binding (Fig. 2A). The *NFX1* gene is highly conserved among eukaryotic species, though little is known about the functions of its homologs.

Differential repressor and activator functions of the NFX1 isoforms on the hTERT *promoter*

Activity in *hTERT* luciferase reporter assays was used as a functional screen for the hits from the yeast two-hybrid screen. Although the *hTERT* promoter is inactive in primary HFKs, expression of either c-Myc or E6 can induce an *hTERT* luciferase reporter construct (Fig. 2B). Transfection of the *NFX1* isoforms alone with the reporter construct had subtle effects, with *NFX1-91* reducing activity to ~60% of background levels, whereas *NFX1-123* had virtually no effect (Fig. 2C). To investigate the activities of the *NFX1* isoforms in the context of an activated promoter, we performed cotransfection experiments with *c-myc* (Fig. 2C). *NFX1-123* strongly coactivated the *hTERT* promoter when cotransfected with *c-myc*, whereas *NFX1-91* robustly repressed induction by *c-myc*. Interestingly, cotransfection of *c-myc* and *E6* cooperatively increased *hTERT* promoter activity, with even higher activity on *NFX1-123* cotransfection. Coexpression of *NFX1-91* reduced *hTERT* activity. These assays indicated that NFX1-91 functioned as a transcrip-

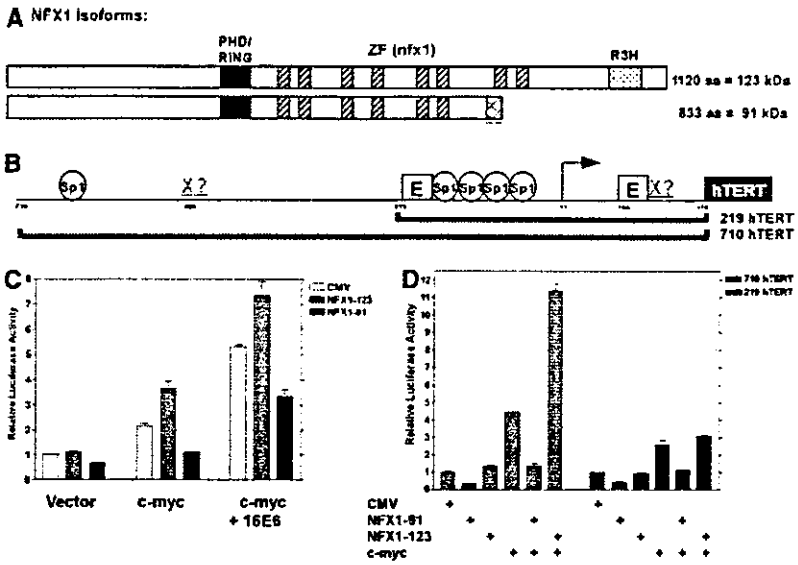

Figure 2. NFX1 isoforms have differential repressive and coactivating functions in hTERT reporter assays. (*A*) Schematic of NFX1 isoforms. The black box represents a PHD/RING finger domain. Multiple hatched boxes indicate NFX1-type zinc-finger domains. The dotted box represents an R3H domain present only in NFX1-123. The cross-hatched box indicates the unique lysine-rich C-terminal domain of NFX1-91. (*B*) Schematic of *hTERT* promoter and the regions included in the luciferase reporter constructs. Several potential SP1-binding sites and E boxes are indicated. Potential NFX1-binding sites are indicated by Xs. (*C*) *hTERT* reporter assay using the 710 hTERT construct in transient transfections in HFKS. The indicated genes were cotransfected with CMV, CMV-NFX1-123, or CMV-NFX1-91. The experiment was done in triplicate. (*D*) *hTERT* reporter assay with two different regions of the promoter in attempt to map the activity of the two NFX1 isoforms. The indicated DNAs were cotransfected with either the 219 hTERT or 710 hTERT reporter construct in HFKs. The data are a representative experiment of three trials done in duplicate.

tional repressor, whereas NFX1-123 appeared to be a coactivator of transcription.

As NFX1 was originally identified in a screen for proteins that bind the X box of MHC class II genes, the presence of similar sequences in the *hTERT* promoter was investigated. The MHC class II X-box sequence is CCTAGCAACAGATG (highly conserved residues are underlined; Song et al. 1994). Sequence scanning of the promoter found two possible X-box-like sequences within the *hTERT* proximal promoter (Fig. 2B). One of these X-box-like sequences (CGTGGGAAGCCCTG) overlapped with the proximal E box and the other (CCTGGGAACAGGTG) lay in the reverse orientation ~400 bp upstream of the transcription start site. In an attempt to map the regions of the *hTERT* promoter bound by the NFX1 isoforms, luciferase assays were performed with a truncated version of the *hTERT* promoter, lacking the upstream putative X box. Activity of the *NFX1* isoforms on an ~800-bp region (710 hTERT) of the *hTERT* promoter was compared with that on a ~300-bp region (219 hTERT; Fig. 2B,D). Interestingly, whereas the coactivation function of *NFX1-123* was clearly apparent with the longer promoter construct, it was greatly diminished with the minimal 219 hTERT promoter. In contrast, the repressive effects of *NFX1-91* could be demonstrated using both promoter constructs. This suggests that these two isoforms may have differential binding affinities for specific elements within the *hTERT* promoter or NFX1-123 may require the presence of additional cofactors bound upstream. It should be noted that c-Myc induction of the 219 *hTERT* construct was significantly lower than that seen with the longer promoter region and this may impact the coactivation ability of NFX1-123.

In summary, luciferase reporter assays indicated that NFX1-91 was a repressor of the *hTERT* promoter, whereas NFX1-123 could coactivate the *hTERT* promoter dependent on sequences located upstream of the minimal 219 promoter fragment.

NFX1-91 binds a putative X box in the proximal promoter of hTERT

To address whether the NFX1 isoforms directly bind the proximal putative X box within the *hTERT* promoter, we generated recombinant His-tagged NFX1 proteins and purified them for use in electrophoretic mobility shift assays (EMSA). The recombinant proteins were truncated to include only the RING finger domain and the NFX1-type zinc fingers, as this is the proposed DNA-binding region (Fig. 3A; Song et al. 1994). Using titrations of recombinant protein, we found that His-NFX1-91 bound and shifted a 48-bp region surrounding the proximal E box and overlapping putative X box much more strongly than similar quantities of His-NFX1-123 (Fig. 3A). The ability of both proteins to bind this region was slightly diminished on mutation of five residues within the X box. Thus, NFX1-91 appeared to have a higher affinity than NFX1-123 for the *hTERT* proximal promoter that appeared to be specific for the X-box region.

Furthermore, the increased affinity of NFX1-91 for binding to the X box appears to reside within the unique C-terminal end of the protein. In another EMSA experiment, a C-terminal peptide (Ac-CASTQKKRSHYMK KIPAH-amide) was sufficient to induce a small shift of the probe DNA that could be dramatically supershifted with a rabbit polyclonal antibody raised to this peptide. Importantly, antibody alone did not induce a similar shift. These data suggest that the unique C terminus of

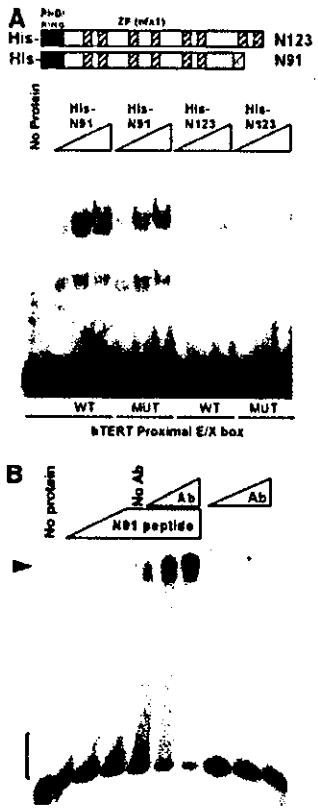

Figure 3. NFX1-91 binds to the proximal putative X box in the hTERT promoter. EMSA assays. (*A*) His-tagged recombinant NFX1 protein fragments, schematically represented at the *top* of the figure, were generated and purified from *E. coli*. Titrations of His-tagged NFX1-91 (His-N91) were able to shift a wild-type (WT) probe of 48 bp surrounding the proximal E box and including the overlapping putative X box. This shift was slightly diminished on mutation of five of the nucleotides within the putative X-box region (MUT). His-tagged NFX1-123 (His-N123) was much less efficient at binding and shifting the same probes. (*B*) A C-terminal peptide of NFX1-91 was sufficient to induce a small shifting (brackets) of the wild-type (WT) X-box DNA probe that could be dramatically supershifted (arrowhead) with titrations of a rabbit polyclonal antibody (Ab) raised to this peptide. The last three lanes demonstrate that antibody alone is unable to induce a similar shift.

NFX1-91 plays a significant role in the affinity of the protein for DNA.

16E6 preferentially binds and destabilizes NFX1-91 rather than NFX1-123

Given the repressive effects of NFX1-91 on the *hTERT* promoter in luciferase assays and its ability to bind the proximal promoter of *hTERT*, an attractive hypothesis emerged that E6/E6-AP might target NFX1-91 for degradation, thereby relieving repression at the *hTERT* promoter. Analysis of the RNA expression levels of the NFX1 isoforms in HFKs expressing E6 or a vector control (LXSN), HeLa (HPV 18-positive cervical carcinoma), C33A (HPV-negative cervical carcinoma), and irrelevant U2OS (osteosarcoma) cells by RT–PCR revealed that the level of expression of the two isoforms did not vary greatly between cell types (Supplementary Fig. S1). As the E6/E6-AP complex reduces expression of its targets by ubiquitin-mediated protein degradation, the effect of E6 expression on NFX1 protein levels was determined. A time course of cells treated with cycloheximide revealed substantial differences in the expression level and half-life of NFX1-91 in HFK/E6 cells compared with HFK/LXSN cells. Although NFX1-91 had an apparently short half-life in the absence of E6, its stability was further decreased in the presence of E6 (Fig. 4A). In contrast, NFX1-123 seemed to be an abundant, stable protein that was largely unaffected by E6 expression (Fig. 4A). To more accurately measure the half-life of NFX1-91 in the presence and absence of E6 expression, we performed

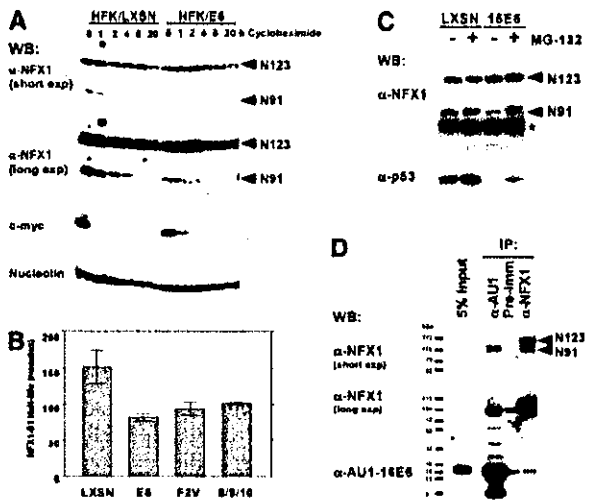

Figure 4. NFX1-91 rather than NFX1-123 is targeted by the E6/E6-AP complex. (*A*) Western blot. HFK/LXSN and HFK/E6 cells were treated with 25 μg/mL cycloheximide for the indicated time points. Lysates were assayed for NFX1 expression levels using an affinity-purified NFX1 antibody. c-Myc is shown as a positive control for a short-lived protein. Nucleolin is a stable protein used as a loading control. (*B*) Half-life of NFX1-91. A pulse-chase experiment to calculate the half-life of NFX1-91 was performed using metabolically labeled HFKs expressing the indicated E6 construct or LXSN vector control. The data presented are the average of two or three independent experiments for each cell line. Error bars represent the standard error of the mean. (*C*) Decreased expression of NFX1-91 seen in E6-expressing HFKs was proteasome dependent. Proteasome inhibition with MG-132 restored NFX1-91 protein levels. NFX1 protein was detected using an IgG-purified NFX1 antibody. The asterisk indicates a nonspecific background band used as a loading control. p53 expression is shown as a control for proteasome inhibition. (*D*) Endogenous NFX1-91 coimmunoprecipitated with AU1-tagged 16E6 in transiently transfected 293T cells. A longer exposure of the NFX1 blot shows the presence of a ubiquitin ladder. The reciprocal IP to precipitate AU1-16E6 with NFX1 antibody did not work.

pulse-chase labeling of HFKs expressing E6 wild-type and mutant proteins followed by immunoprecipitation with an antibody specific for NFX1-91. As shown in Figure 4B, E6 expression reduced the half-life of NFX1-91 ~46%, from 2.5 h in vector control cells to 1.3 h in E6-expressing cells. Expression of two E6 mutants, F2V and 8S/9A/10T, which induce telomerase but do not target p53 for degradation despite their ability to bind E6-AP, caused reductions in NFX1-91 half-life similar to that found with E6 wild type (Fig. 4B). The reduction of NFX1-91 protein levels in E6-expressing HFKs was proteasome dependent, as treatment with the proteasome inhibitor MG-132 restored NFX1-91 protein levels to that found in vector-transduced HFKs (Fig. 4C). Although E6 interacted with NFX1-123 in the yeast two-hybrid screen and in in vitro binding assays (data not shown), E6 preferentially bound to NFX1-91 and not to NFX1-123 in coimmunoprecipitations from 293Ts transiently transfected with AU1-tagged E6 (Fig. 4D). Although we were unable to confirm the interaction with the reciprocal coimmunoprecipitation using NFX1 rabbit polyclonal antibody, the sum of the other protein expression data and binding data strongly suggested that NFX1-91 was a novel target of the E6/E6-AP E3 ubiquitin ligase complex.

NFX1-91 is highly ubiquitinated in the presence of E6

As E6 appeared to preferentially bind and destabilize NFX1-91 in a proteasome-dependent manner, we examined the ubiquitination status of NFX1-91 versus NFX1-123 in cell lysates. For these experiments, Flag-tagged versions of the NFX1 isoforms were transiently transfected into 293T cells in order to specifically immunoprecipitate the two different isoforms. Although expression of both isoforms was driven by the CMV promoter, NFX1-123 was much more highly expressed than NFX1-91 on transfection (Fig. 5A), consistent with the differences in protein stability previously observed (Fig. 4). In addition, Western blots with a ubiquitin antibody revealed a strong ladder signal only in the Flag-tagged NFX1-91 immunoprecipitations (Fig. 5B), indicating NFX1-91 was highly ubiquitinated whereas NFX1-123 was not, thus explaining their different half-lives. As this experiment was conducted in cells lacking E6 expression, it indicated that NFX1-91 protein levels, much like p53 protein levels, were regulated in a proteasome-dependent manner even in the absence of E6. To determine if E6 expression could influence the degree of NFX1-91 ubiquitination, we repeated the experiment in HFK/LXSN and HFK/E6 cells with endogenous NFX1 protein. We developed specific antibodies to separately immunoprecipitate each isoform of NFX1. As seen in Figure 5C, ubiquitinated NFX1-91 was much more prevalent in HFK/E6 cells than in HFK/LXSN cells, and NFX1-123 did not appear to be ubiquitinated. A longer exposure of the ubiquitin Western blot shown in Figure 5C indicated that there is some ubiquitinated NFX1-91 in HFK/LXSN cells (data not shown). Therefore, as we saw in Figure 5B with the 293T cells, an E6-independent means of ubiquitinating NFX1-91 seems to exist. Significantly, the higher levels of ubiquitinated NFX1-91 detected in HFK/E6 cells directly correlated with the decreased stability of NFX1-91 protein in the presence of E6 (Fig. 4), strongly supporting the hypothesis that NFX1-91 is a target of E6/E6-AP-mediated degradation.

In vivo evidence for NFX1-91 as a repressor of hTERT

Thus far, luciferase reporter assays suggested NFX1-91 functions as a repressor at the *hTERT* promoter and protein expression data indicated that the E6/E6-AP complex could destabilize NFX1-91 protein, suggestive of a relief of repression mechanism for E6-mediated *hTERT* induction. To address directly whether NFX1-91 functions as a transcriptional repressor at the endogenous *hTERT* promoter, we reduced *NFX1-91* expression using stable shRNA expression in HFKs. We constructed an

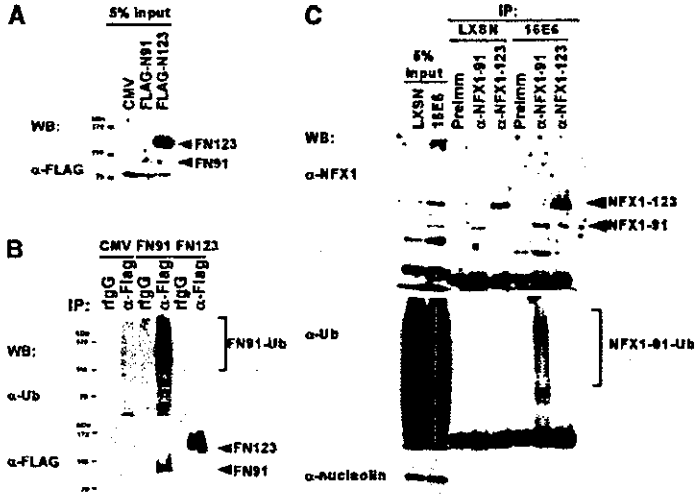

Figure 5. NFX1-91 is ubiquitinated by E6-independent and E6-dependent means. (*A*) Western blot. 293Ts were transiently transfected with Flag-tagged forms of NFX1. Cells were treated with MG-132 prior to lysis. Proteins were detected with rabbit anti-Flag antibody. (*B*) The lysates shown in *A* were immunoprecipitated with rabbit IgG (rIgG) or a rabbit anti-Flag antibody (α-Flag). Western blots with a mouse anti-ubiquitin antibody indicate that NFX1-91 was highly ubiquitinated, whereas NFX1-123 was not. (*C*) Endogenous NFX1 isoforms were immunoprecipitated from HFK/LXSN and HFK/E6 cells with antibodies specific for each isoform or with preimmune serum. Anti-NFX1 detects both NFX1 isoforms and anti-ubiquitin detects ubiquitinated proteins. Nucleolin is a loading control for the input lysates.

shRNA construct targeted to the unique 3' untranslated region (3'UTR) of *NFX1-91*. The NFX1-91 shRNA (n91sh) reduced NFX1-91 protein levels in HFKs while not affecting NFX1-123 protein levels (Fig. 6A). This was accompanied by derepression of the *hTERT* promoter even in cells lacking E6 expression (Fig. 6B). The previously characterized E6-AP sh1 (esh1) construct (Fig. 1) was included as a negative control. When the shRNA-expressing cell lines were subsequently transduced with empty vector or E6, the E6 cells with reduced *NFX1-91* expression had a greater than twofold increased expression of *hTERT* as demonstrated by RT–PCR and TRAP assay (Fig. 6C,D). Furthermore, the lower telomerase activity resulting from decreasing *E6-AP* expression in HFK/E6 cells correlated with increased NFX1-91 protein levels, decreased detection of ubiquitinated NFX1-91, and an increased half-life of NFX1-91 from 1.2 to 1.7 h (Fig. 6E,F). These data indicated that NFX1-91 functions as a transcriptional repressor at the endogenous *hTERT* promoter in HFKs and that the activity of the *hTERT* promoter increased in response to decreased NFX1-91 protein expression levels, either by shRNA expression or by E6/E6-AP-mediated ubiquitination and degradation.

To further validate the role of NFX1-91 in regulation of telomerase activity, we examined the impact of reduced *NFX1-91* expression on the lifespan of cells in culture. The cells presented in Figure 6C were continually passaged, splitting 1:3 as needed for ~2 mo (Fig. 7A). As cells divide, the telomeres of cells that do not express telomerase continually shorten and the telomere structure becomes disrupted (Hahn et al. 1999b; Masutomi et al. 2003). Cells respond to the chromosome ends as though they are a double-stranded DNA break and generally attempt to repair the exposed chromosome ends or initiate a senescent arrest (Espejel and Blasco 2002; d'Adda di Fagagna et al. 2003; Takai et al. 2003). Senescent cells can be identified by a change in cellular morphology and with a marker for senescent-associated (SA)-β-galactosidase activity (Dimri et al. 1995). As expected, there were fewer senescent cells present in the E6-expressing population compared with the vector control population (Fig. 7B). Furthermore, about half of the E6 cells with reduced *E6-AP* expression stained positive for SA-β-galactosidase and appeared large and flattened with many vacuoles (Fig. 7B,C). In contrast, the n91sh-expressing cells, even in the absence of E6 expression, were more rounded like rapidly dividing cells and displayed reduced SA-β-galactosidase staining. The percent of cells exhibiting SA-β-galactosidase activity (Fig. 7C) was inversely related to the telomerase activity detected at earlier passages (Fig. 6C,D). Additionally, the cells with increased telomerase activity had increased proliferative capacity in culture, as indicated by the number of population doublings each cell line achieved during the same number of days in culture (Fig. 7A,B). Therefore, reduction of NFX1-91 protein levels either via reduction of mRNA levels with shRNA or via E6/E6-AP-mediated ubiquitination and degradation was sufficient to induce *hTERT* expression and delay senescent growth arrest in primary human epithelial cells. Conversely, reduced *E6-AP* expression prohibits E6-mediated telomerase induction and lifespan extension through the stabilization of the *hTERT* transcriptional repressor, NFX1-91.

Although delayed senescence correlated well with in-

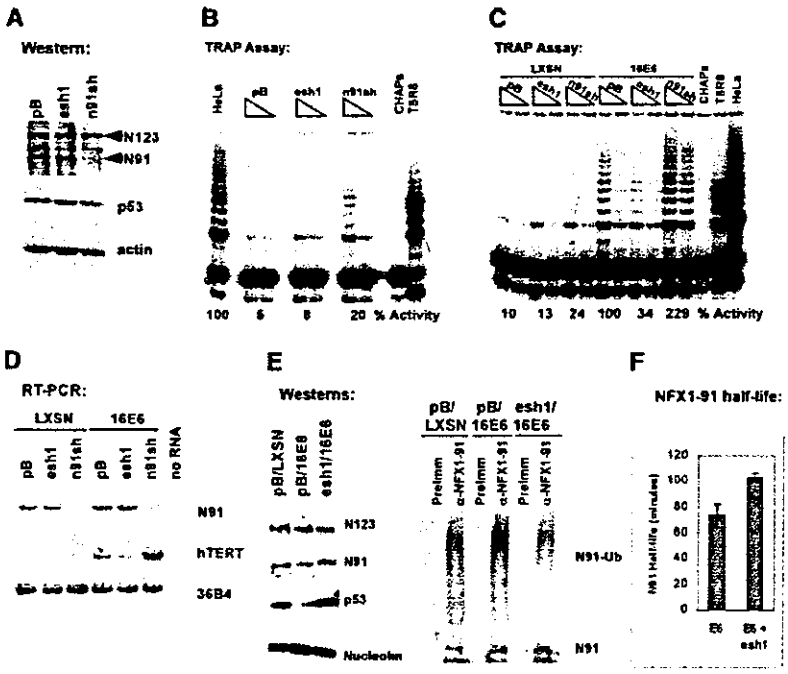

Figure 6. Knockdown of *NFX1-91* expression using shRNAs derepresses the endogenous hTERT gene in HFKs. (*A*) Western blot. NFX1-91 protein expression was reduced with expression of n91sh. (*B*) TRAP assay. Lysates from HFKs transduced with pB, esh1, or n91sh were analyzed for telomerase activity. Two micrograms and 0.5 μg of each lysate were used in the TRAP reactions. HeLa is a positive control lysate (0.2 μg). The percent of TRAP activity is presented relative to that in the HeLa lane. (*C*) TRAP assay. The cells shown in *A* and *B* were subsequently transduced with LXSN or LXSN-16E6. Lysates were examined for telomerase activity. The percent of TRAP activity is presented relative to that in the pB/LXSN-16E6 cells. (*D*) RT–PCR. Expression of *hTERT* and *NFX1-91* was examined in RNA extracts from the cells in *C*. (*E*) Western blots. In vivo ubiquitination assays were performed as in Figure 4 with HFKs transduced with pB/LXSN, pB/E6, or esh1/E6. The blots on the *left* show protein expression in the lysates used for IP (5% input). The blots on the *right* show levels of ubiquitinated NFX1-91 immunoprecipitated from the cells. (*F*) NFX1-91 half-life. A pulse-chase analysis of NFX1-91 protein half-life in two independent lines of HFKs expressing E6 and empty vector (E6) or E6 and the esh1 shRNA (E6 + esh1) found an increase in protein half-life from 1.2 to 1.7 h. Error bars represent the standard deviation of two independent experiments.

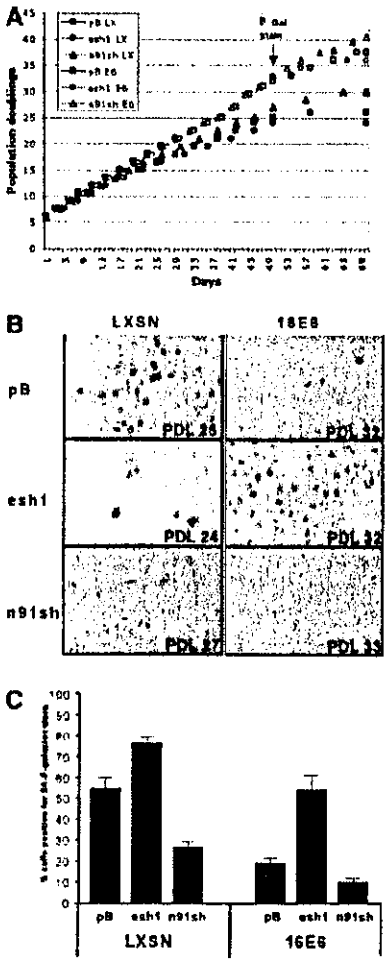

Figure 7. Senescence-associated β-galactosidase activity in late-passage HFKs. (*A*) Growth curves. HFKs expressing the indicated shRNA and LXSN (closed symbols) or E6 (open symbols) were grown in culture for ~2 mo. The pBabe vector control is indicated by black squares, the esh1 cells are represented by red circles, and the n91sh cells are indicated by green triangles. The time point at which cells were stained for SA-β-galactosidase is indicated. (*B*) Micrographs of SA-β-galactosidase staining of late-passage HFKs expressing the indicated shRNA and LXSN empty vector or LXSN-16E6. The blue staining indicates senescent cells. (*C*) Quantitation of SA-β-galactosidase staining seen in *B*. The data represent the average percent of blue cells counted in four different fields at 100× magnification. The error bars indicate the standard error of the mean.

creased telomerase activity, we were unable to observe any significant differences in the telomere lengths of the cells presented in Figures 6 and 7 (data not shown). Telomere-length Southern blots indicated that telomeres continued to shorten regardless of the level of telomerase expression (data not shown). Previous studies of mortal cultures of E6-expressing epithelial cells have also observed a lack of telomere-length maintenance despite the induction of telomerase expression (Kiyono et al. 1998). This may indicate that telomerase is acting only at the shortest telomeres to maintain proper telomere structure rather than increasing telomere lengths (Hemann et al. 2001; Masutomi et al. 2003). We propose that the continued proliferation and lack of β-galactosidase-positive cells in the telomerase-expressing cells more accurately reflects the maintenance of proper telomere structure than the gross changes in telomere length observed by telomere-length Southern blot analysis.

Discussion

Induction of telomerase appears to be a common and requisite event in the immortalization and transformation of many cell types. In our studies to determine how HPV-16 E6 induces telomerase in epithelial cells, we have identified NFX1-91 as a cellular repressor of the human *hTERT* promoter both in vivo and in vitro that is destabilized by the E6/E6-AP complex. Previous work by our group and others suggested a correlation between the ability of E6 to bind its cellular partner E6-AP and the induction of telomerase (Table 1). The E6/E6-AP complex is proposed to target many different cellular proteins for ubiquitination and degradation (Fehrmann and Laimins 2003; Scheffner and Whitaker 2003), including a large family of PDZ domain-containing proteins that are membrane-associated guanylate kinases, or MAGUKs (Thomas et al. 2001). The PDZ-binding domain of E6, encompassing the four C-terminal amino acids, has been shown to be required for transformation of established rodent cells by E6 (Kiyono et al. 1997) but is dispensable for immortalization by E6 (Kiyono et al. 1998). Therefore it is unlikely that any of the PDZ-containing proteins targeted by E6 are involved in telomerase activation. Here, we provide direct evidence of the requirement for E6-AP expression for E6-mediated *hTERT* induction (Fig. 1). These findings prompted the search for novel targets of the E6/E6-AP complex that might function as transcriptional repressors at the *hTERT* promoter.

Identified as an interactor of E6/E6-AP in a yeast two-hybrid screen, NFX1, a known transcriptional repressor, seemed an ideal candidate for a hypothesized telomerase repressor degraded on E6 expression in epithelial cells. This gene actually encoded two splice variants, *NFX1-123* and *NFX1-91*, with opposing activities in *hTERT* reporter assays (Fig. 2). NFX1-123 strongly coactivated with c-Myc at the *hTERT* promoter, whereas NFX1-91 repressed the activity of the promoter. Coexpression of E6 with either isoform increased the activity of the promoter, suggesting that E6 could co-operatively activate *hTERT* with NFX1-123 and could decrease the repressive effects of NFX1-91. Furthermore, coimmunoprecipitation experiments indicated that the E6/E6-AP complex preferentially interacted with the NFX1-91 isoform and stimulated its ubiquitination and degradation (Figs. 4, 5). We propose that E6/E6-AP induces telomerase by destabilizing the *hTERT* transcriptional repressor, NFX1-91. In support of this hypothesis, decreased expression of NFX1-91 using shRNAs was sufficient to induce *hTERT* expression and telomerase activity in primary human

epithelial cells (Fig. 6). Unfortunately, as seen in Figure 5A, NFX1-91 did not overexpress well despite repeated attempts (data not shown); therefore, we were unable to demonstrate repression of the *hTERT* promoter in response to increased expression of NFX1-91.

A recent study by Lin and Elledge (2003) found three tumor suppressor/oncogene pathways involved in *hTERT* repression; these were Mad1, menin, and SIP1 (Lin and Elledge 2003). Reduced expression of any one of these *hTERT* repressors was sufficient to induce *hTERT* expression in previously telomerase-negative cells. It is striking that these repressors do not seem redundant; oncogenic stimulation that abrogates any one of them is sufficient to relieve repression. Therefore, it is not implausible that, despite the identification of several *hTERT* repressor proteins, E6/E6AP specifically targets only one transcriptional repressor, NFX1-91, to induce *hTERT* expression. It is likely that future investigations will reveal different cell-type specificities for *hTERT* repressors and variability in the responsiveness of different cell types to perturbations of these repressors.

In reporter assays, E6 seems to require an intact proximal E box in the *hTERT* promoter for activity (Gewin and Galloway 2001; Veldman et al. 2003). Although c-Myc levels do not appear to change dramatically on E6 expression (Gewin and Galloway 2001; Veldman et al. 2001), two groups have recently found by chromatin immunoprecipitation that c-Myc is bound to the *hTERT* promoter in E6-expressing cells (Baege et al. 2002; Veldman et al. 2003). McMurray and McCance (2003) suggest that in E6-expressing cells, c-Myc replaces USF1 and USF2 at the *hTERT* promoter, and Veldman et al. (2003) find that c-Myc is bound to the *hTERT* promoter in both hTERT-negative E7-expressing cells and hTERT-positive E6-expressing cells. In unpublished data from our lab, we also found that c-Myc was bound at the *hTERT* promoter in both vector control and E6-expressing HFKs. Therefore, binding of c-Myc to the *hTERT* promoter was not sufficient for promoter activity. Because our data suggested that disruption of the NFX1-91 repressor of telomerase was involved in the activation of telomerase, it is interesting to note that a putative NFX1-binding site overlaps with the proximal E box. EMSA experiments indicate that a recombinant fragment of NFX1-91 binds to this region with significantly higher affinity than a largely similar fragment of NFX1-123. These data help to clarify how NFX1-91, an unstable protein expressed at much lower levels than NFX1-123, could repress the hTERT promoter in the presence of abundant NFX1-123 protein levels. Our future efforts will determine whether either of the two NFX1 isoforms is differentially bound to this site in vivo in E6-expressing cells.

Although reporter assays indicated that the NFX1-123 isoform could function as a coactivator of the *hTERT* promoter, this isoform is highly expressed, stable, and apparently unaffected by E6 expression. NFX1-123, with its two additional zinc fingers and R3H domain, most likely exists in a very different conformation than NFX1-91 that is not recognized by the E6/E6-AP complex. As structure relates to function, this likely influences the DNA-binding affinity, as seen by EMSA in Figure 3, and functional consequences of DNA binding for NFX1-123. Whether the coactivation function of NFX1-123 is an integral part of E6-mediated *hTERT* induction remains to be tested. Interestingly, the instability and repressive functions of NFX1-91 as well as its higher affinity for the X-box-like sequences in the *hTERT* promoter appeared to reside in the unique lysine-rich C terminus.

Although we propose that the targeting of NFX1-91 for ubiquitination and degradation by the E6/E6-AP complex is involved in the induction of telomerase in epithelial cells, this may be only one step in the process of robustly inducing *hTERT* expression by E6. Previous data have indicated that induction of *hTERT* by HPV-16 E6 may involve epigenetic phenomenon such as histone acetylation or chromatin remodeling (Klingelhutz et al. 1996; Kiyono et al. 1998; Baege et al. 2002). Many transcriptional regulators function by covalently modifying the histones associated with the nearby chromatin. Recently, it has been demonstrated that ubiquitination of histone H2B may influence the methylation status of histone H3 and thereby mark the chromatin as active for transcription (Muratani and Tansey 2003). Given that E6/E6-AP and the NFX1 RING finger possess E3 ubiquitin ligase activity, it will be interesting to investigate the ubiquitination status of histone H2B at the *hTERT* promoter in E6-expressing cells.

Ubiquitination and transcriptional activation may also be intimately linked in a developing model involving the ubiquitination and degradation of transcriptional activators bound at promoters (Muratani and Tansey 2003). The activators rely on E3 ligase activity found within coactivators to license their activity and link increased protein turnover with clearing of the promoter for subsequent rounds of transcription. In fact, the Skp2 ubiquitin ligase is a coactivator of c-Myc at the cyclin D2 promoter and loss of Skp2 can stabilize c-Myc protein levels and reduce transactivation of c-Myc-responsive promoters (Kim et al. 2003; von der Lehr et al. 2003). Although c-Myc steady-state levels do not change on E6 expression, it is possible that this is a result of balanced c-Myc induction (Kinoshita et al. 1997) and increased degradation (Gross-Mesilaty et al. 1998). E6/E6-AP has been demonstrated to ubiquitinate c-Myc in vitro and in vivo (Gross-Mesilaty et al. 1998). In fact, E6 has been found at the *hTERT* promoter and E6 can immunoprecipitate c-Myc from cell lysates (Veldman et al. 2003). Although we have been unable to demonstrate an interaction between c-Myc and E6, it remains possible that these proteins do interact in a complex at the *hTERT* promoter. An attractive synthesis of this model with our data is that E6/E6-AP may function at multiple levels to induce *hTERT*. First, the E6/E6-AP complex may target NFX1-91 for increased turnover to derepress the promoter; then, an E3 ligase may ubiquitinate and activate the c-Myc bound at the *hTERT* promoter. This theory and the possible role of the E3 ligase activity of the NFX1 isoforms or the E6/E6-AP complex to ubiquitinate either histone H2B or c-Myc remains to be tested.

In summary, we have identified NFX1-91 as a novel

cellular repressor of the *hTERT* promoter in primary human epithelial cells. Significantly, interference with its expression is sufficient to induce telomerase expression and extend the lifespan of primary epithelial cells, thus making NFX1-91 an important new target of transformation mechanisms.

Materials and methods

Cell culture

Primary human keratinocytes (HFKs) were derived from neonatal foreskins and grown in EpiLife medium supplemented with calcium chloride (60 μM) and human keratinocyte growth supplement (Cascade Biologics). 293T, HeLa, C33A, and U20S cells were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL) containing 10% fetal calf serum (FCS) and penicillin-streptomycin. SF9 cells were grown in SF 900 II serum-free medium (GIBCO-BRL) containing 5% FCS and gentamycin.

Plasmids

The E6-AP and NFX1-91 shRNA constructs were generated by previously described methods (Paddison et al. 2002; Grandori et al. 2003; Smith et al. 2003). Briefly, oligos containing the 26- to 29-nt shRNA sequence were used in PCR reactions to clone the U6 or H1 RNA polymerase III promoter upstream into a pBabe-puro-based vector. The E6-AP shRNA targeted sequences were as follows: esh1 5′-CTAATAGAACGCTACTACCACCAGT TAAC-3′, esh2 5′-AGAGATTGTTGAAGGCCATCACGTAT GCC-3′, and esh3 5′-ACAATGAAGAAGATGATGAAGAGC CCATC-3′. The n91sh construct was targeted to the 3′UTR region (5′-TGTGGAACCAGCCCAACTGCCCATCAGTCAA-3′). The NFX1-123 isoform was PCR cloned from a HeLa cell cDNA library and the NFX1-91 isoform was cloned from a fetal brain cDNA library. These genes (with and without a Flag tag) were subsequently inserted by restriction digest or via the GATEWAY recombination-based system (Invitrogen) into a CMV-based vector for transient transfections. The AU1 tag (DTYRYI) was fused to the N terminus of 16E6 by PCR and subsequently cloned into a CMV-based vector using the GATEWAY system (Invitrogen). The pBabe-c-myc vector was obtained from Carla Grandori. The pGL3-based *hTERT* luciferase reporter constructs have been previously described (Gewin and Galloway 2001). The His-tagged NFX1 isoforms were generated by PCR cloning the indicated fragments into the BamHI site of the pET16b vector (Novagen) in frame with multiple histidine residues.

Retroviral infections

Retroviruses were produced either in established viral producer cell lines (PA317 or PG13) or in 293Ts by a transient VSV-G-pseudotyped virus production protocol as previously described (Bartz and Vodicka 1997). Cells were infected at ~60% confluence in 6-cm tissue culture plates. Twenty-four hours after infection, cells were expanded to 10-cm plates and allowed to adhere to the plate for 4–24 h before adding selective media. HFKs were selected in 0.5 μg/mL puromycin or 50 μg/mL G418. Cells expressing shRNAs were maintained in puromycin-containing media.

Northern blotting

Total cellular RNA was isolated using the RNeasy kit (QIAGEN). From 20 to 60 μg of total RNA was electrophoresed on 1% agarose-formaldehyde gels, transferred to Hybond-N membranes (Amersham), and hybridized to ^{32}P-labeled probes. The E6-AP probe was generated by random primer labeling (Roche) a 440-bp XhoI fragment of the E6-AP gene. The 36B4 loading control probe has been described previously (Kiyono et al. 1998).

Yeast two-hybrid screen

The yeast two-hybrid screen was performed using the Matchmaker GAL4 two-hybrid system (Clontech). A catalytically defective E6-AP mutant (C833A) was fused to the GAL4 DNA-binding domain in the pGBT9 plasmid. The 16E6 gene was also cloned into this plasmid under the control of the ADH2 promoter. Both a HeLa cell cDNA library and a fetal brain cDNA library were fused to the GAL4 activation domain in the pGAD and pACT2 plasmids, respectively. Clones that grew on selective media were subjected to a secondary screen for β-galactosidase activity. Full-length clones were obtained by 5′ RACE (rapid amplification of cDNA ends) using the Marathon cDNA amplification kit (Clontech).

Luciferase assays

Luciferase assays were performed as previously described (Gewin and Galloway 2001). Briefly, HFKs were grown to 50%–60% confluence in six-well plates and transfected with a pGL3-based hTERT reporter plasmid (710 hTERT or 219 hTERT) and CMV- or pBabe-based expression constructs. A total of 2 μg of DNA was transfected into each well using a 1:3 DNA:FuGENE (Roche) ratio. Cells were incubated for 24 h after transfection, rinsed in phosphate-buffered saline (PBS), and lysed in the well by freeze-thawing in 100 μL of reporter lysis buffer (Promega). Cell debris was removed by centrifugation. Luminescence was quantitated in 10 μL of each lysate on mixing with luciferase assay buffer (Promega) in a Monolight 2010 luminometer. Each experiment was done several times in duplicate and normalized for total protein concentration.

Recombinant protein purification

pET16b constructs expressing His-tagged NFX1 isoforms were transformed into Codon (+) DE3 cells and grown in 100-mL cultures to an $O.D._{600}$ of ~0.6. Protein expression was induced for 2 h at room temperature with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Proteins were extracted from inclusion bodies using denaturing conditions (8 M urea, 10 mM Tris-Cl, 100 mM NaH_2PO_4, 20 mM β-mercaptoethanol, 10% glycerol, 1% NP-40, 300 mM sodium chloride, 20 μM zinc chloride adjusted to pH 8) and purified on Ni-NTA beads (Qiagen). After washing with lysis buffer adjusted to pH 6.3, the beads and bound recombinant proteins were dialyzed stepwise to remove urea and NP-40 prior to elution with increasing concentrations of imidazole (100–250 mM).

EMSA

Probes were generated by radiolabeling, annealing, and gel purifying complementary oligonucleotides containing sequences from the *hTERT* promoter. Sequences correspond to 15 bp upstream of the proximal E box (CACGTG) and extend 27 bp downstream. Five base pairs were changed (GGAAGCCCTG to TGGGGCCCGA) to generate the X-box mutant probe. Titrations of recombinant proteins (1–6 μL) were incubated with 5000 cpm of radiolabeled probes in 40-μL binding reactions containing 25 mM HEPES (pH 7.5), 5% glycerol, 5 μM zinc chloride, 5 mM magnesium chloride, 50 mM potassium chloride, and 0.1 mg of bovine serum albumin per milliliter. The peptide shifts

were performed identically using titrations (20–100 ng) of peptide and supershifted with titrations (1–4 μg) of affinity-purified rabbit polyclonal antibody raised to this peptide. Reactions were run on 4.5% polyacrylamide gels in 25 mM HEPES (pH 7.5).

RT–PCR

RNA was isolated from cells using the RNeasy kit (QIAGEN). cDNA was synthesized using random hexamers and the Superscript II reverse transcriptase system (Invitrogen). RNase H (2 units) was added to the reactions and incubated for 20 min at 37°C. Expression of hTERT, NFX1-123, NFX1-91, and 36B4 was detected by PCR using either ethidium bromide staining or [^{32}P]α-dCTP incorporation and autoradiography. Sequences of hTERT and 36B4 primers are previously described (Gewin and Galloway 2001). NFX1-123 primers are (F) 5′-TCCCTCCCAT GAACAGAGAC-3′ and (R) 5′-TTTCAAGCACACCTGTCAG C-3′. NFX1-91 primers are (F) 5′-TTACCCTCCAGTTCCCT GTG-3′ and (R) 5′-CATGCGTGTGCAGGTATCTT-3′.

Telomerase activity

Telomerase activity was detected using the radioisotopic detection method of the TRAPeze telomerase detection kit (Serologicals Corporation, Chemicon International).

Generation of rabbit polyclonal antibodies

Rabbit polyclonal antibodies were generated to recognize both NFX1 isoforms (α-NFX1) and each isoform, NFX1-123 (α-NFX1-123) and NFX1-91 (α-NFX1-91), individually. Recombinant full-length NFX1-123 was generated in SF9 cells using a baculovirus system. Briefly, NFX1-123 was cloned into the pVIC1 plasmid in which NFX1 was fused to a chitin-binding domain with an intervening intein sequence as described previously (Chong et al. 1997; Pradhan et al. 1999). This construct was transfected into SF9 cells using the BaculoGold system (Pharmingen) to generate infectious baculovirus. SF9s infected with baculovirus were harvested and lysed in buffer M (50 mM Tris-HCl at pH 7.4, 1 mM EDTA, 500 mM sodium chloride, and COMPLETE protease inhibitor tablet). The SF9 protein lysate was precleared on cellulose resin and subsequently purified with chitin beads. Full-length NFX1-123 was cleaved from the chitin-binding domain in two volumes of buffer M plus 0.5% Tween-20, 4 mM dithiothreitol, and 5% glycerol incubated at 16°C overnight and used to generate a rabbit polyclonal antibody to recognize both NFX1 isoforms. The NFX1-123-specific antibody was raised to a recombinant His-tagged C-terminal fragment of NFX1-123 (amino acids 932–1120) expressed in and purified from *Escherichia coli*. The NFX1-91-specific antibody was raised to a C-terminal peptide (Ac-CASTQKKRSHYMKKIPAH-amide) generated by BIOSOURCE.

Western blotting

Whole-cell lysates were prepared for Western blotting by trypsinizing cells, washing with PBS, and resuspending in WE16th lysis buffer (50 mM Tris-HCl at pH 7.5, 250 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 20% glycerol, 10 μM zinc chloride, 2 mM dithiothreitol, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a COMPLETE protease inhibitor tablet [Roche]). Lysates were then sonicated and clarified by centrifugation. Protein concentrations were determined by using the DC protein assay (Bio-Rad). Protein lysates were electrophoresed on SDS–polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blots were performed with goat anti-actin (Santa Cruz Biotechnology, I-19), mouse anti-p53 (Oncogene Science, Ab6), mouse anti-c-myc (Santa Cruz Biotechnology, C-33), mouse anti-nucleolin (Santa Cruz Biotechnology, C-23), rabbit anti-AU1 (Bethyl), mouse anti-Flag (Sigma, M2), and mouse anti-ubiquitin (Covance, P4G7). Rabbit polyclonal NFX1, NFX1-123, and NFX1-91 antibodies are described earlier.

Proteasome inhibition and cycloheximide treatment

In proteasome inhibition experiments, cells were treated with 10–20 μM MG-132 (Calbiochem) or an equal volume of dimethyl sulfoxide (solvent control) for 4 h at 37°C. For half-life analysis, HFKs were treated with 25 μM cycloheximide (Calbiochem) and harvested in WE16th lysis buffer at the indicated time points.

Pulse-chase labeling of cells and protein half-life calculations

Keratinocytes were metabolically labeled with Express Label [^{35}S]cysteine-[^{35}S]methionine (DuPont NEN) in 154XP medium (Cascade Biologics) for 1 h. Cells were chased for various periods of time with EpiLife keratinocyte medium, washed two times in cold PBS, and lysed on ice for 30 min in RIPA buffer + 1 mM EDTA. Following a brief sonication, the lysates were frozen in liquid nitrogen and stored at −80°C until completion of the time course. Lysates were thawed quickly and insoluble debris was pelleted. Lysates were subsequently precleared with protein A agarose prior to immunoprecipitation with preimmune or anti-NFX1-91 rabbit serum. After incubation with protein A agarose beads, immunocomplexes were washed once with RIPA buffer, twice with high-salt buffer (500 mM sodium chloride, 50 mM Tris at pH 8, 1% NP-40), and once more with RIPA buffer. The beads were boiled in sample buffer and electrophoresed on a 6% SDS–polyacrylamide gel. The gels were dried and subjected to phosphorimaging (Molecular Dynamics). Band intensities were measured using ImageQuant.

Immunoprecipitations

293Ts at 60% confluence in 15-cm plates were transfected with 20 μg of CMV-AU1-16E6 with FuGENE (Roche). Twenty-four hours later, cells were treated with 10 μM MG-132 (Calbiochem) for 2 h prior to harvest. 293Ts were harvested for immunoprecipitation by rinsing with cold PBS. Cells were pelleted and resuspended in an NP-40 lysis buffer (1× PBS, 0.5% NP-40, 10% glycerol, 10 μM zinc chloride, 2 mM dithiothreitol, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a COMPLETE protease inhibitor tablet [Roche]). Cells were lysed by quick freezing in liquid nitrogen and thawing in a room-temperature water bath. Cell debris was pelleted at 14,000 rpm for 15 min and lysates were precleared by rotating at 4°C with 50 μL of protein A agarose (Roche). After centrifugation to remove the beads, lysates were incubated with the appropriate antibody for 1–2 h at 4°C and purified by adding protein A agarose and rotating for another hour at 4°C. Immunocomplexes were washed three times with lysis buffer and eluted by heating for 10 min at 70°C in 2× sample buffer. Elutions were electrophoresed on NuPAGE 4%–12% Tris-Bis gradient gels (Invitrogen) to resolve both AU1-tagged 16E6 and NFX1 isoforms and Western blotted as described earlier.

In vivo ubiquitination assay

Cells were treated with 20 μM MG-132 for 2 h prior to harvest. Lysates were prepared by trypsinizing cells and washing with

PBS. Cell pellets were resuspended in 2% SDS in PBS and boiled for 10 min, then diluted in five to six volumes of 1% Triton X-100 in PBS. The lysates were sonicated on ice and clarified by centrifugation followed by preclearing with protein A agarose for 30 min at 4°C. The lysate was subsequently divided for individual immunoprecipitations with the appropriate antibody and incubated at 4°C for 1–2 h. Preimmune rabbit serum and normal rabbit IgG (Santa Cruz Biotechnology) were used as negative controls. To precipitate bound proteins, we added protein A agarose to each immunoprecipitation and rotated it for 1 h at 4°C. Bound proteins were washed three times with PBS and eluted by boiling for 5 min in 2× sample buffer. Elutions were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting as described earlier.

SA-β-galactosidase staining

Cells were washed three times in PBS containing 1 mM magnesium chloride and fixed in 3% formaldehyde in PBS for 5 min at room temperature. After three washes in PBS (pH 6.0), cells were stained in PBS (pH 6.0) containing 1 mM magnesium chloride, 0.12 mM potassium ferricyanide, 0.12 mM potassium ferrocyanide, and 1 mM X-GAL at 37°C overnight.

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