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14-3-3 proteins and the p53 family : a study in keratinocytes

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14-3-3 proteins and the p53 family

a study in keratinocytes

Maarten Niemantsverdriet

14-3-3 proteins and the p53 family

a study in keratinocytes

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Thesis outline

14-3-3 proteins constitute a family of highly homologous, conserved proteins, abundantly expressed in all eukaryotic organisms studied so far. There are seven human 14-3-3 isoforms (β , γ , ϵ , η , σ , τ and ζ), all expressed from different genes. By their interaction with binding partners, 14-3-3 proteins regulate many cellular processes that are important to cell biology, such as signal transduction, transcriptional control, apoptosis, cell-cycle control, differentiation and stress response. The general properties of 14-3-3 proteins are discussed in **chapter 1**. Because most of the studies presented in this thesis were performed in keratinocyte cells, the role of 14-3-3 proteins in these cells is described in more detail.

In recent years more and more direct connections between 14-3-3 proteins and members of the p53 family of transcription factors have emerged in literature. The p53 family consists of three genes, p53, p63 and p73 which are involved in the same or similar cellular processes as 14-3-3 proteins. An introduction into the p53 family and the direct relationships between this family and 14-3-3 proteins are discussed in **chapter 2**. Also in this chapter, the role of these proteins in keratinocytes is emphasized.

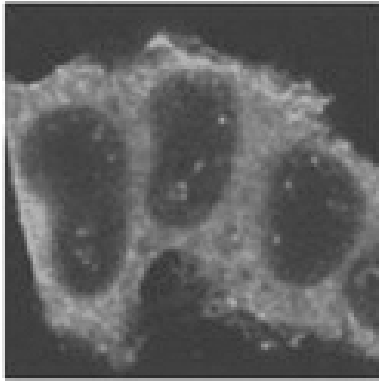
Since human 14-3-3 proteins are highly homologous, have similar protein structures and biochemical properties, as discussed in chapter 1, the different 14-3-3 isoforms are often assumed to all behave in a similar way. Nevertheless, isoform-specific 14-3-3 actions have been observed. In **chapter 3**, isoform specific functions in tumorigenesis of one of the 14-3-3 proteins, 14-3-3 ζ , were studied after specific downregulation of this isoform in an immortal human keratinocyte cell line.

14-3-3 proteins are localized in the cytoplasm of a cell, but also in the nucleus albeit at a lower concentration. Differences in nucleocytoplasmic shuttling of 14-3-3 proteins might reflect a molecular basis for isoform-specific biological specialisation. Therefore, the differences in subcellular distribution and dynamic localization

between 14-3-3 σ and 14-3-3 ζ were studied in varying living mammalian cells in **chapter 4**.

Indications accumulate that the 14-3-3 σ isoform is unique among the 14-3-3 proteins. In contrast to other 14-3-3 isoforms which are ubiquitously expressed, 14-3-3 σ is expressed only in epithelial cells and it has a crucial role in the differentiation of keratinocytes. In cell types other than keratinocytes several links between p53 family members and especially the 14-3-3 σ isoform have been found. In colorectal cancer cells 14-3-3 σ is induced by p53 following irradiation, which is required for proper G2 arrest. In **chapter 5**, the possible involvement of 14-3-3 σ transactivation or stabilization in radiation induced G2 arrest in keratinocytes in a p53 regulated keratinocyte cell line is studied. In **chapter 6** the retention of a double GFP protein is monitored in p53 transfected and permeabilized keratinocyte cells. Finally, in **chapter 7**, a widely used method to detect different p73 splice variants is critically assessed.

Chapter 1



14-3-3 proteins

Introduction

14-3-3 proteins are a family of highly conserved, acidic proteins with a size of approximately 30 kD, abundantly expressed in a wide range of organisms and tissues. The unusual name was given during a systematic attempt to classify bovine brain proteins. The 14-3-3 proteins eluted in the 14th fraction of bovine brain extract and were found on positions 3.3 of subsequent starch-gel electrophoresis by Moore and Perez (Moore & Perez, 1967). All eukaryotic organisms express multiple 14-3-3 isoforms, ranging from 2 in yeast and 7 in mammalian cells up to 15 in plants. Despite this genetic diversity, 14-3-3 proteins display an amazing level of sequence conservation between isoforms. For example, the 14-3-3 isoform BMH1 from *Saccharomyces cerevisiae* (yeast) is, at the amino acid level, approximately 70% similar compared to the human 14-3-3 ϵ isoform (Wang & Shakes, 1996).

Mammalian 14-3-3 isoforms were initially named α , β , γ , δ , ϵ , η and ζ after the reversed phase HPLC separation pattern of 14-3-3 proteins isolated from bovine brain (Ichimura et al., 1988). However, Aitken *et al.* showed that the α and δ forms identified by Ichimura *et al.* represent the phosphorylated forms of β and ζ respectively (Aitken, 1995). The 14-3-3 τ isoform (sometimes called θ) was originally identified as a T-cell specific isoform (Nielsen, 1991), but it was shown that other cell types also express this variant (Aitken, 2006). The last mammalian isoform discovered was the σ isoform (also called stratifin or HME1), which is expressed only in epithelial cells (Leffers et al., 1993; Prasad et al., 1992). So, in total, mammalian (including human) cells express 7 different 14-3-3 isoforms called $\beta(\alpha)$, γ , ϵ , η , σ , $\tau(\theta)$ and $\zeta(\delta)$.

The high degree of sequence conservation and the abundance of 14-3-3 proteins in all eukaryotic organisms studied until now, suggest that they are of fundamental biological importance for organisms ranging from yeast to humans. More than 20 years after the discovery of 14-3-3 proteins, were they were identified simply as abundant brain proteins with unknown function, the first experimental evidence that 14-3-3 proteins play an important role in cell biology emerged when they were found to interact with proto-oncogene and signalling proteins (Aitken et al., 1990; Reuther et al., 1994; Toker et al., 1990).

Over 300 14-3-3 interacting proteins have been identified until now (Aitken, 2002; Fu et al., 2000; Pozuelo Rubio et al., 2004; van Hemert et al., 2001), however, for most binding partners it is still unclear whether binding is specific for one isoform or if it can be achieved by several or all 14-3-3 isoforms. Among the 14-3-3 binding partners are proteins involved in major cellular processes such as cell cycle control, intracellular signalling, stress response, apoptosis, transcriptional regulation, cellular metabolism, cytoskeletal integrity, cell motility, adhesion and differentiation. Accordingly, a prominent role for 14-3-3 proteins in these processes has been accentuated by experimental studies; these are reviewed in (Aitken, 2006; Aitken et al., 1995; Berg et al., 2003; Burbelo & Hall, 1995; Darling et al., 2005; Fu et al., 2000; Hermeking, 2003; Hermeking & Benzinger, 2006; Lee & Lozano, 2006; Masters et al., 2002; Porter et al., 2006; Reuther & Pendergast, 1996; Rosenquist, 2003; Tzivion & Avruch, 2002; Tzivion et al., 2006; Tzivion et al., 2001).

Structure, dimerization and binding

Amino-acid sequence comparison of 14-3-3 proteins from various organisms shows that 14-3-3 proteins consists of five highly conserved blocks separated by less conserved regions and suggests that 14-3-3 proteins in all organisms share a very similar structure (Wang & Shakes, 1996). The first two human 14-3-3 X-ray crystal structures solved were those of the ζ and the τ isoforms (Liu et al., 1995; Xiao et al., 1995). These studies revealed almost identical dimeric structures. Each 14-3-3 monomer is composed of nine α -helices (referred to as α A- α I) organised in an antiparallel fashion, creating an L-shaped structure (see figure 1). The four N-terminal helices mediate dimerization and form a planar surface to which the five C-terminal helices have a rectangular orientation, creating a U-shaped (clamp-like) dimer. The crystal structures of all human 14-3-3 isoforms have now been solved; in general the structures are very similar, which is not surprising considering the high degree of sequence conservation (Gardino et al., 2006). Of the 7 human 14-3-3 isoforms, the 14-3-3 σ form shows the most deviant structure (Wilker et al., 2005). Accordingly, experimental data accumulate that 14-3-3 σ shows much more isoform-

specific activities than other 14-3-3 isoforms (Dellambra et al., 2000; Hermeking, 2003; Hermeking & Benzinger, 2006; Lee & Lozano, 2006).

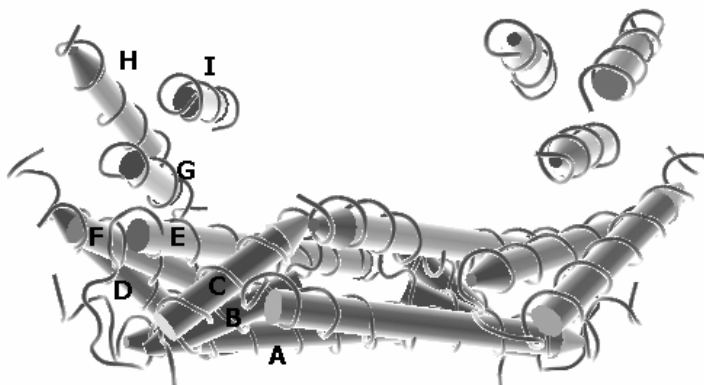


Figure 1. Structure of a 14-3-3 (zeta) dimer, organization of the nine α -helices in the left monomer is indicated (A-I).

The dimer contact residues are highly conserved among human 14-3-3 isoforms (Wang & Shakes, 1996), this allows some isoforms to form heterodimers as well as homodimers. The human 14-3-3 ϵ variant even forms heterodimers preferentially, while the σ and γ variants normally only form homodimers (Benzinger et al., 2005; Chaudhri et al., 2003; Gardino et al., 2006; Wilker et al., 2005). The difference in propensity to homo- or heterodimerize is thought to be determined by difference in salt-bridges between the 14-3-3 isoforms (Gardino et al., 2006). The crystal structure of 14-3-3 σ revealed that the σ isoform contains a unique salt bridge, alternative to the salt bridges in the other isoforms, this likely accounts for the homodimeric character of this isoform (Wilker et al., 2005).

A combination of mutational analysis and co-crystallization studies of 14-3-3 proteins with binding peptides showed that a target binding groove is formed in the 14-3-3 dimer, composed of residues from both N-terminal and C-terminal regions of 14-3-3 proteins (Ichimura et al., 1995; Liu et al., 1995; Wang et al., 1998; Xiao et al., 1995). The C-terminal α -helices form the walls of the U-shaped 14-3-3 dimer, creating the binding groove between the two L-shaped monomers. The interior of each monomer consists of two α -helices with charged and polar amino acids (α C and α E) and two with hydrophobic amino acids (α G and α I), forming a highly conserved

concave amphipathic groove that acts as a binding channel, binding specific motifs in the target protein (Petosa et al., 1998; Wang et al., 1998). Therefore, each monomer is capable of binding a specific motif in a target protein, allowing a 14-3-3 dimer to bind two motifs simultaneously, this can be either two motifs in the same target protein or binding two target proteins, one motif each (Liu et al., 1995; Obsil et al., 2001; Rittinger et al., 1999; Xiao et al., 1995; Yaffe et al., 1997). See figure 2 for a schematic representation of a 14-3-3 dimer.

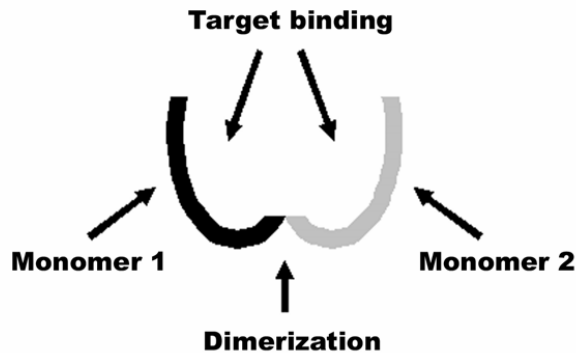


Figure 2. Schematic representation of a 14-3-3 dimer. 14-3-3 monomers are shown in black (monomer 1) and grey (monomer 2).

14-3-3 target binding is usually regulated by phosphorylation of a potential 14-3-3 binding motif in the target protein. The early findings that 14-3-3 target proteins tryptophan and tyrosine hydroxylase (Furukawa et al., 1993; Ichimura et al., 1988) and Raf and Bcr (Michaud et al., 1995) required phosphorylation for 14-3-3 binding stimulated a more specified study to determine the specific requirements for 14-3-3 binding (Muslin et al., 1996). In this study, Muslin *et al.* identified a 14-3-3 binding motif consisting of RSxpSxP, where pS represents phosphoserine and x any amino acid. Later, using phosphopeptide libraries, evidence was provided that there are two distinct 14-3-3 binding motifs, RSxpSxP (mode 1) and Rx ϕ pxSxP (mode 2) where pS represents either phosphoserine or phosphothreonine, ϕ an aromatic or aliphatic amino acid and x any amino acid (Rittinger et al., 1999; Yaffe et al., 1997). In addition to these well-defined motifs recognized by all 14-3-3 isoforms, 14-3-3 proteins are capable of binding variations of the modes 1 and 2 motifs (Tzivion et al., 2001) and other less well characterized phosphorylated motifs in target proteins

might also be recognized by 14-3-3 proteins (Coblitz et al., 2006). Most interactions between 14-3-3 proteins and binding partners depend on phosphorylation of the target, however, several interactions have also been described that do not require phosphorylation at all, these are summarized in (Gardino et al., 2006).

Modes of action

Although for most 14-3-3 binding partners the function of 14-3-3 binding is not clear yet, some general mechanisms by which 14-3-3 association regulates the function of its targets have emerged (Hermeking, 2003; Tzivion & Avruch, 2002; Tzivion et al., 2001; van Hemert et al., 2001). A single 14-3-3 binding partner can be regulated by only one or by a combination of these mechanisms. The mechanisms can be arranged into five general modes of action (see figure 3):

1) 14-3-3 binding can alter the subcellular localization of the target protein and sequester it (normally) in the cytoplasm. This is the most commonly described mode of action for 14-3-3 proteins. There are two mechanisms by which this may be accomplished: First, 14-3-3 proteins can facilitate the re-localization of the target protein from the nucleus to the cytoplasm via a nuclear export sequence in the 14-3-3 protein, and second, binding of 14-3-3 may cover up a nuclear import sequence in the target protein and reduce the nuclear import rate. Both mechanisms result in the sequestration of the target protein in the cytoplasm. Proteins regulated in this manner include: Cdc25C (Dalal et al., 1999; Kumagai & Dunphy, 1999), Cdc2/cyclinB1 complex (Chan et al., 1999), p27 (Fujita et al., 2002) and FKHRL1 (Brunet et al., 1999).

2) 14-3-3 binding can prevent the interaction of the target protein with other proteins. For example, 14-3-3 binding to the pro-apoptotic protein BAD prevents binding to the anti-apoptotic protein Bcl2 (Datta et al., 2000; Hsu et al., 1997; Zha et al., 1996). In this case, the result of 14-3-3 association is inhibition of apoptosis because 14-3-3 binding to Bad relieves the Bcl2 protein, which is then free to perform its survival promoting function. Another example of this mode of action is

14-3-3 binding to IRS-I, which prevents activation of PI-3K by IRS-I (Kosaki et al., 1998).

3) 14-3-3 proteins can act as a scaffold to bridge two targets. 14-3-3 proteins can bind two targets at the same time and stimulate their interaction. This mode of action has been described for the interactions of Raf with Bcr (Braselmann & McCormick, 1995), A20 (Vincenz & Dixit, 1996) and PKC (Van Der Hoeven et al., 2000).

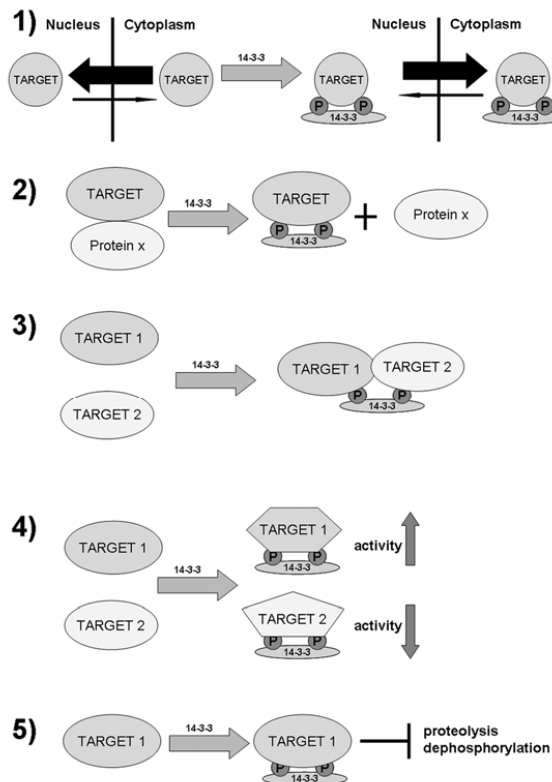


Figure 3. 14-3-3 modes of action. See text for details.

4) Binding of 14-3-3 proteins can alter the activity of the target protein and inhibit or enhance its activity. A few examples are: 14-3-3 association enhances tryptophan and tyrosine hydroxylase activities (Ichimura et al., 1988) and p53 DNA binding capacity (Waterman et al., 1998) and 14-3-3 association inhibits ASK-I activity (Liu et al., 2001; Zhang et al., 1999) and RGS activity (Benzing et al., 2000).

5) 14-3-3 binding can protect a target protein from modifications such as dephosphorylation and modifications signalling for proteolysis. 14-3-3 binding to Raf (Dent et al., 1995; Thorson et al., 1998), histones (Chen & Wagner, 1994) and BAD (Chiang et al., 2001) prevents dephosphorylation of the targets. Association of 14-3-3 with p53 (Yang et al., 2003), plant nitrate reductase (Weiner & Kaiser, 1999) and other 14-3-3 targets in *Arabidopsis* (Cotelle et al., 2000) protects the partners from proteolysis.

14-3-3 proteins and keratinocytes

The outermost layer of the human skin is the epidermis, which is mainly composed of keratinocyte cells. Early 14-3-3 research indicated that 14-3-3 proteins might have a specific role in keratinocytes. They are capable of enhancing protein kinase C (PKC) activity, which is involved in the regulation of keratinocyte differentiation (Dellambra et al., 1995). Keratinocytes form a stratified epithelium consisting of several distinct layers, named basal layer, spinous layer, granular layer and the cornified layer (Fuchs & Raghavan, 2002). They are constantly renewed through division of stem cells in the basal layer, they move upwards towards the outside edge of the skin as they (terminally) differentiate and give rise to the various layers of the epidermis, resulting in lifeless flattened squames forming the cornified layer, which ultimately detach from the skin (Alonso & Fuchs, 2003; Roop, 1995). Each layer is characterized by a specific protein profile of differentiation markers such as keratins, involucrin, loricrin, filaggrin and sprr proteins, classifying the state of differentiation (Alonso & Fuchs, 2003; Cabral et al., 2001; Fuchs & Green, 1980; Fuchs & Raghavan, 2002; Roop, 1995). Keratins are major structural proteins, they form a family of approximately 30 members expressed in a differentiation specific manner in epithelial cells (Fuchs & Green, 1980; Steinert & Roop, 1988). Defects in keratin functioning can result in various skin defects which are typical for the epidermal layer expressing the malfunctioning keratin (Roop, 1995).

Different keratins have been identified as 14-3-3 binding partners by several independent groups (Kim et al., 2006; Ku et al., 2002; Liao & Omary, 1996; Omary &

Ku, 2006; Pozuelo Rubio et al., 2004). It is not entirely clear which 14-3-3 isoforms bind which keratins and how isoform specific this binding is and for most interactions the function of 14-3-3 binding remains to be elucidated. Kim *et. al.* showed that the interaction between keratin 17 and 14-3-3 σ likely plays an important role in epidermal wound healing (Kim et al., 2006). However, the isoform-specificity has to be clarified since other (yeast) 14-3-3 proteins have also been shown to bind human keratin 17 (Pozuelo Rubio et al., 2004).

14-3-3 σ expression is restricted to epithelial cells (Leffers et al., 1993), which suggest an isoform specific role in these types of cells, it was suggested that this role might be differentiation of epithelial cells. Dellambra *et al.* showed that 14-3-3 σ is expressed at relatively low levels in basal keratinocyte cells, at high levels at the moment of commitment to terminal differentiation and again at low levels when differentiation is completed (figure 4). 14-3-3 σ is absolutely required for differentiation of human epidermal keratinocytes since downregulation of 14-3-3 σ prevents differentiation of primary human keratinocytes by forcing them into the stem cell compartment, this allows the cells to escape from replicative senescence and become immortal (Dellambra et al., 2000).

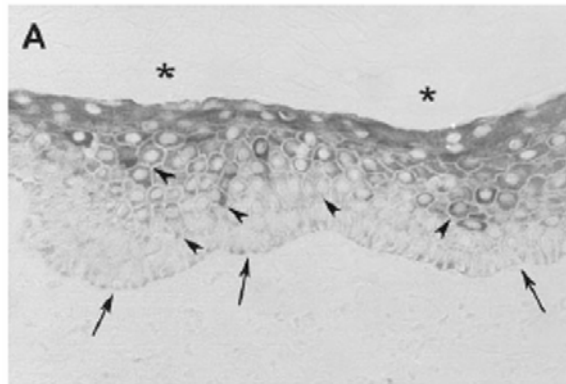


Figure 4. Expression of 14-3-3 σ in human skin. Human skin was stained with a 14-3-3 σ -specific antibody. 14-3-3 σ is barely detectable in the basal layer (arrows), increasingly expressed in suprabasal layers (arrowheads) and undetectable in the cornified layer (asterisks). Picture adapted from (Dellambra et al., 2000).

A fine balance between extracellular matrix (ECM) synthesis and degradation is of crucial importance for the maintenance of the skin's structural integrity and for repair of injured skin. Excessive wound healing is characterized by increased deposition of ECM which often results in fibrotic conditions. Differentiated keratinocytes excrete 14-3-3 σ ; this stimulates expression of extracellular matrix degrading proteins such as MMP-1, MMP3 and collagenase-1 (and others) by dermal fibroblasts. Excreted 14-3-3 σ also downregulates expression of structural ECM proteins in fibroblasts, together this indicates that 14-3-3 σ excreted by keratinocytes plays a major role in structuring the skin and preventing fibrotic conditions after skin injury (Ghaffari et al., 2006; Ghahary et al., 2004).

Taken together, 14-3-3 proteins have clearly been shown to be involved in keratinocyte physiology. Especially the 14-3-3 σ isoform has a crucial role in keratinocyte differentiation and maintenance of skin structure.

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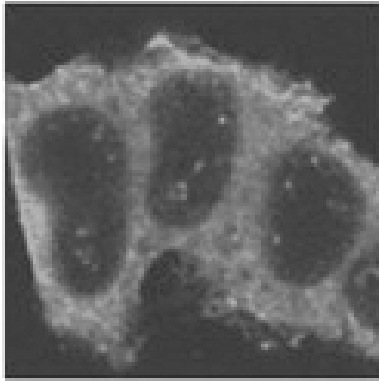
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Chapter 2



14-3-3 proteins and the p53 family

Introduction

14-3-3 proteins are involved in many cellular processes, including processes regulated by the p53 family of transcription factors. Indirect links comprise 14-3-3 proteins associating with various transcriptional targets of p53 family members (Benzinger et al., 2005; Fu et al., 2000; Pozuelo Rubio et al., 2004; van Hemert et al., 2001). However, more direct connections have also been observed, these will be discussed in this chapter. The direct relationship between 14-3-3 proteins and members of the p53 family mainly focuses on 14-3-3 σ because all p53 family members are capable of transactivating this isoform. However, multiple 14-3-3 isoforms can bind p53 family members and regulate their activity. So, p53 family members directly regulate 14-3-3 and vice versa 14-3-3 isoforms directly regulate p53 family members.

The p53 family

The p53 family was named after p53, the first family member, discovered in 1979 (DeLeo et al., 1979). The p53 gene encodes a sequence specific transcription factor, activated in response to stress signals such as radiation and other types of genotoxic damage. Increased levels of p53 prevent replication of damaged cells by enhancing the expression of genes inducing cell-cycle arrest and programmed cell death (apoptosis). P53 has become one of the most intensively studied genes in cancer biology, it was found that the gene is mutated or inactivated in over 50% of all human cancers and p53 is now considered the most important tumor-suppressor in mammals (Bates & Vousden, 1996; Horn & Vousden, 2004; Lane, 1994; Morgan & Kastan, 1997; Prives & Hall, 1999; Steele et al., 1998; Vogelstein et al., 2000). The role as tumor-suppressor is accentuated by p53-null mouse models, which show that mice lacking p53 spontaneously develop various types of tumors and die of cancer two to twelve months after birth (Donehower et al., 1992; Jacks et al., 1994).

Considering the importance of p53 in cancer research perhaps surprisingly, the two p53 homologues p63 and p73 were discovered 20 years after the discovery of

p53. P73 was identified in 1997 (Kaghad et al., 1997) and later several research groups independently identified p63 (Osada et al., 1998; Schmale & Bamberger, 1997; Senoo et al., 1998; Tan et al., 2001; Trink et al., 1998; Yang et al., 1998), which resulted in several alternative names for p63 (KET, p40, p73L, p53CP and NBP). The three p53 family members are very homologous and all three contain a conserved transactivation domain (TA), a DNA binding domain (DBD) and an oligomerization domain (OD) (also called tetramerization domain) (Yang & McKeon, 2000). The highest sequence similarity is found in the DNA binding domain, where almost all tumor related mutations in p53 are found, this region is over 60% identical in all three family members. In experimental systems, p63 and p73 showed behaviour mimicking p53, such as binding p53 target sites, transactivating p53 target genes and inducing p53-like phenotypes (Ishida et al., 2000; Jost et al., 1997; Osada et al., 1998; Shimada et al., 1999; Yang et al., 1998; Zhu et al., 1998). However, despite the sequence conservation and the similarities in behaviour in experimental systems, the functions of p63 and p73 appear to differ from those of p53 *in vivo*. In contrast to p53 null-mice, severe developmental abnormalities are observed in p63 and p73 null mice (figure 1). P73 null mice are much smaller than p53 null or wild-type mice and show neurological, pheromonal and inflammatory defects but they lack spontaneous tumors (Yang et al., 2000). P63 null-mice show severe defects in limb and epithelial development. Because the skin of these mice is not developed properly (explained in more detail below), they die from dehydration soon after birth which eliminates the possibility of investigating the tumor-suppressing capacity of p63 in this system (Mills et al., 1999; Yang et al., 1999).

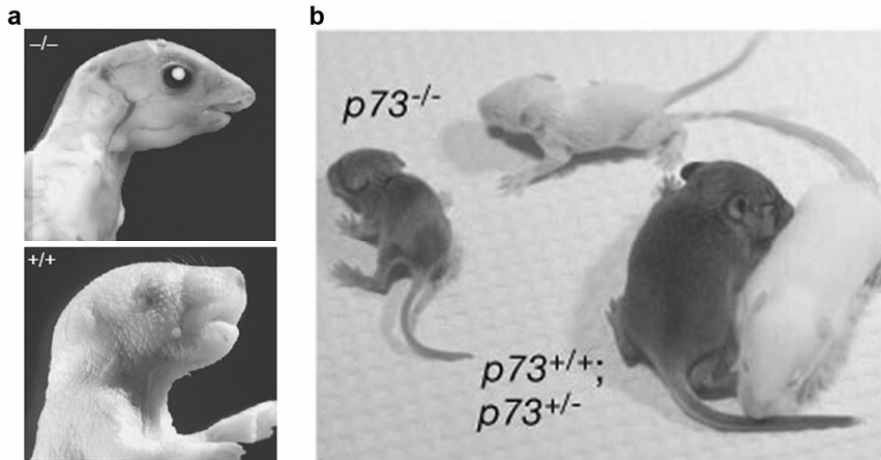


Figure 1. P63 and p73 deficient mice show severe developmental abnormalities. (a) p63 $-/-$ mice on postnatal day 1 show severe skin and limb defects. (b) p73 $-/-$ mice at postnatal day 10 are significantly smaller than p73 $+/+$ and p73 $+/-$ mice. Pictures were adapted from Yang et al., 1999 (a) and Yang et al., 2000 (b).

Until 2005, p53 expression was usually regarded as simple; a single promoter directs the synthesis of one protein which transactivates a specific set of target genes. The p63 and p73 genes were found to be much more complex, giving rise to proteins that either resemble p53 function but also proteins that counteract the function of p53. P63 and p73 are both expressed from two independent promoters and present a variety of alternative splice-variants, which results in a complex array of p63 and p73 proteins (Yang & McKeon, 2000). See figure 2 for a schematic representation of the p53 family members.

Transcription of both p63 and p73 can start from either exon 1 or from an additional exon 3' (located between exon 3 and 4). P63 or p73 proteins expressed from exon 3' lack the N-terminal part of the protein which houses the transactivation domain, these proteins are usually incapable of transactivation. In fact, these forms, named ΔN variants, normally act as dominant negatives for p63, p73 and p53 proteins that do contain a transactivation domain (TA forms) and counteract their function (De Laurenzi & Melino, 2000; Irwin & Kaelin, 2001; Levrero et al., 2000; Marin & Kaelin, 2000; Yang et al., 2002; Yang & McKeon, 2000). In addition to the ΔN forms, other variations in the N-terminal regions have also been found, reviewed in (Moll & Slade, 2004).

P53 family members assemble into tetramers (by means of the oligomerization domain) to form an active complex. Because the oligomerization domains are conserved in all p53 family members, formation of heterotetramers is possible, although homo-tetramerization is favoured (Davison et al., 1999; De Laurenzi & Melino, 2000). Tetramers built up solely from ΔN isoforms compete for specific DNA sites in the promoter regions of target genes and prevent DNA binding and thus transactivation by (active) p53, TAp63 or TAp73 tetramers. In addition, because all ΔN p63 and p73 forms still contain the oligomerization domain, they can be incorporated into a tetrameric complex containing TA forms, which inactivates the tetramer (Grob et al., 2001; Marin & Kaelin, 2000; Yang & McKeon, 2000). Although ΔN p63 or p73 show repressor activity in almost all experiments, there are exceptions that indicate that some ΔN forms might sometimes have a positive role in transactivation (Liu et al., 2004).

In addition to alternative use of promoters, p63 and p73 also give rise to a variety of C-terminal splice variants. The full-length C-terminal splice-variants, designated p63 α and p73 α both contain a sterile (sometimes called steric) α -motif (SAM domain) and a transcription inhibitory domain (TID) in addition to the transactivation domain, the DNA binding domain and the oligomerization domain also present in p53 (De Laurenzi & Melino, 2000; Yang et al., 2002; Yang & McKeon, 2000). P63 expresses three C-terminal splice variants, α , β and γ . The p63 gene consists of 15 exons, compared to the full-length α variant, the β form lacks exon 13 which is part of the SAM domain. P63 γ lacks exons 11 to 14, containing the complete SAM domain and the TID domain. Both the SAM and TID domains usually inhibit transactivation of p63 target genes although the effect of these domains varies between targets. Because regions that are not found in p53 are spliced out in p63 β and γ , these isoforms structurally resemble p53 more than p63 α . In contrast to p63 α , experimental evidence shows that TAp63 β and TAp63 γ transactivate p53 inducible genes at levels similar to p53 and ectopically expressed TAp63 β and TAp63 γ induce apoptosis or cell cycle arrest, similar to p53 (Osada et al., 1998; Yang et al., 1998).

P73 splicing is similar to p63 but even more complex, at least eight p73 proteins alternative to the full-length α form are generated by splicing of the C-terminus, named β , γ , δ , ϵ , ϕ , ζ , η and η_1 (De Laurenzi & Melino, 2000; Ishimoto et

al., 2002; Kaghad et al., 1997; Moll & Slade, 2004; Stiewe et al., 2002; Yang et al., 2000). The most important splice-variants are schematically shown in figure 2. Like p63, TAp73 splice-variants structurally resembling p53 more than p73α generally show more p53-like characteristics.

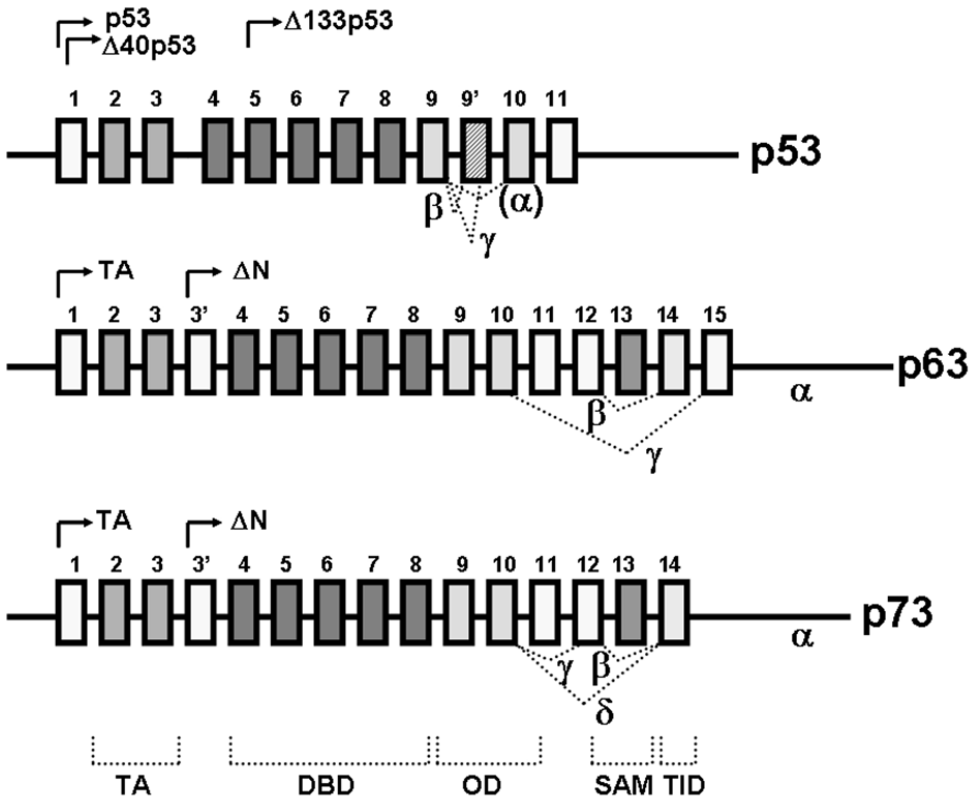


Figure 2. Schematic representation of p53 family structure. The main structural domains, transactivation domain (TA), DNA binding domain (DBD), oligomerization domain (OD), sterile alpha motif (SAM) and the transcription inhibitory domain (TID) are indicated below the structures. Arrows represent promoter start sites. Splicing is indicated by dotted lines, only the most important splice variants are indicated.

The identification of C-terminal splice variants and use of alternative promoters for p63 and p73 stimulated the search for alternative p53 variants. Indeed, the more sophisticated techniques currently available allowed the identification of p53 variants other than the commonly described p53 protein and also for p53, C-terminal splice variants and use of alternative promoters has been

detected (Bourdon et al., 2005). The human p53 gene encodes at least nine different p53 isoforms named p53, p53 β , p53 γ , Δ 133p53, Δ 133p53 β , Δ 133p53 γ , Δ 40p53, Δ 40p53 β and Δ 40p53 γ (figure 2), they are expressed in normal human tissues, in a tissue-dependent manner. Early experiments indicate that p53 variants show activities comparable to corresponding p63 or p73 variants. For example, p53 β binds differentially to promoters and enhances expression of p53 target genes in a promoter-dependent manner and Δ 133p53 shows dominant-negative capacities towards (normal) p53. Considering the biological importance of Δ N and C-terminal p63 and p73 variants, it is likely that the novel p53 variants exhibit very important biological functions. However, because these novel p53 variants were discovered only recently and information about their functions is exceptionally limited, these variants will not be discussed further and when p53 is mentioned it indicates full-length p53.

From accumulating experimental data, it is becoming clear that p63 and p73 show important functional differences to each other and to p53. Whereas p53 remains the principle tumor suppressor in mammalian cells, gene specific roles for p63 include epithelial cell maintenance, development of skin, limbs, breast urothelium and prostate. Specific roles for p73 include neuron survival, hippocampal neurogenesis, cerebral fluid homeostasis, reviewed in Yang et al., 2002. However, there is no doubt that p53 family members also have similar biological functions. In fact, p63 and p73 appear to be essential for p53 functioning in response to DNA damage because the family members enhance the binding of p53 to target gene promoters (Flores et al., 2002). Also, genes have been found that can be transactivated by (splice-variants of) all three family members, these include p21, bax, mdm2, cyclin-G, gadd45, IGFBP (De Laurenzi et al., 1998; Jost et al., 1997; Kaghad et al., 1997; Osada et al., 1998; Yang et al., 1998; Zhu et al., 1998). All p53 family members are also capable of transactivating 14-3-3 σ ; this will be discussed in more detail in the next paragraph.

14-3-3 proteins and the p53 family

Serial analysis of gene expression (SAGE) showed that 14-3-3 σ is upregulated after DNA-damage induction in colorectal cancer cell lines expressing wild-type p53. It was also shown that 14-3-3 σ is not upregulated following DNA damage in colorectal cancer cells expressing mutant p53, suggesting that p53 is directly involved in the transactivation of 14-3-3 σ . Ectopically overexpressed wild-type p53 is capable of increasing 14-3-3 σ mRNA levels without DNA damage in the same cell type. The p53 mediated induction of 14-3-3 σ is required for maintaining a stable cell cycle arrest in G2 phase after DNA damage. Exogenous expression of 14-3-3 σ results in G2 arrest in colorectal cells directly (Hermeking et al., 1997). Cells deficient for 14-3-3 σ fail to sequester the cdc2/cylinB1 complex and die of mitotic catastrophe in response to DNA damage (Chan et al., 1999). Transactivation of 14-3-3 σ by p53 is mediated through a functional p53-binding site, located in the promoter region of the 14-3-3 σ gene, 1.8 kilobases upstream of the 14-3-3 σ transcription start site (Hermeking et al., 1997).

The same motif binds Δ Np63 α , however as expected from the lack of a transactivation domain, this protein represses transcription of 14-3-3 σ (Westfall et al., 2003). Because Δ Np63 α protein is capable of binding the p53-binding sites in the 14-3-3 σ promoter and TAp63 splice-variants contain exactly the same DNA binding domain it is likely that the latter forms are capable of transactivating 14-3-3 σ . Experimental support for this came from the finding that in p53-negative hepatocytes, both TAp63 and 14-3-3 σ are induced following DNA damage, suggesting that in this case TAp63 is responsible for 14-3-3 σ transactivation (Fomenkov et al., 2004).

TAp73 forms also transactivate the 14-3-3 σ gene, when ectopically expressed, the TAp73 α and β variants even showed 3 to 6 times higher transactivation of 14-3-3 σ than p53 itself (Zhu et al., 1998). In contrast to what is regarded the normal function of Δ N splice variants, also Δ Np73 β and Δ Np73 γ are capable of inducing 14-3-3 σ , in fact, in MCF-7 cells, Δ Np73 β was more active in inducing 14-3-3 σ than the TA form of p73 β (Liu et al., 2004). The authors of this paper suggest that the Δ Np73 variants form an alternative activation domain in the unique N-terminal residues of

the protein, which is responsible for the transactivation of some of the p53 target genes. 14-3-3 σ appears to be exceptionally sensitive for activation by this domain since it was the only gene in this study that was induced more effectively by Δ Np73 β than by TAp73 β (Liu et al., 2004).

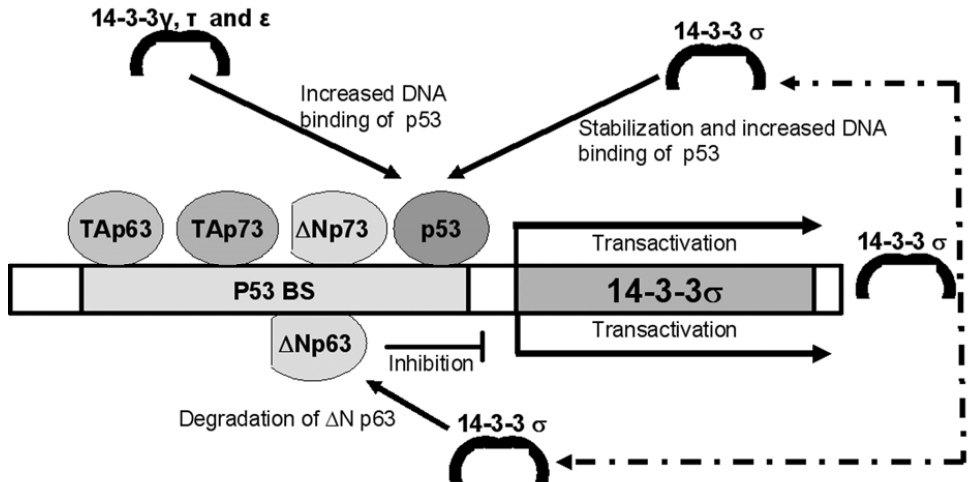


Figure 3. Direct relationships between 14-3-3 proteins and the p53 family. After DNA damage, p53 family members bind a p53 binding-site (p53 BS) in the 14-3-3 σ promoter. P53, TAp63, TAp73 and Δ Np73 variants transactivate 14-3-3 σ which in return binds p53 itself, hereby stabilizing p53, increasing DNA binding and thus transcription by p53, creating a positive feedback loop. DNA damage creates a 14-3-3 binding-site in p53 where other 14-3-3 isoforms can bind p53, which enhances transactivation by increasing the DNA binding capacity of p53. Binding of Δ Np63 to the p53 binding-site inhibits transactivation of 14-3-3 σ and association of Δ Np63 with 14-3-3 σ results in degradation of Δ Np63. See text for details.

P53 is constitutively phosphorylated at serines 376 and 378 and after DNA damage induced by γ -irradiation, p53 is dephosphorylated at serine 376, which creates a 14-3-3 binding-site centred by serine 378. This motif can bind 14-3-3 γ , 14-3-3 τ and 14-3-3 ϵ , which enhances p53 function by increasing sequence specific binding to DNA, resulting in higher transactivation potential (Stavridi et al., 2001; Waterman et al., 1998).

P53 stability is regulated by MDM2, which is a ubiquitin ligase that decreases the half-life of p53 by marking p53 with ubiquitin leading to degradation by the

ubiquitin-proteasome pathway (Iwakuma & Lozano, 2003). 14-3-3 σ itself binds p53 in response to DNA damage; this binding has a positive effect on p53 stability and increases its half-life by preventing degradation mediated by MDM2. Increased levels of 14-3-3 σ also lead to destabilization of MDM2 itself. In addition, 14-3-3 σ binding to p53 promotes tetramerization, resulting in increased transcriptional activity, similar to binding of 14-3-3 γ , 14-3-3 τ and 14-3-3 ϵ to p53 (Yang et al., 2003). So, following DNA-damage, 14-3-3 σ modulates the activity of p53, which can in turn result in transactivation of 14-3-3 σ itself, thus creating a positive-feedback loop. The opposite appears to be true for Δ Np63 α , because it lacks a transactivation domain, this protein inhibits transactivation of 14-3-3 σ , in response to DNA damage 14-3-3 σ binds to Δ Np63 α and transports it from the nucleus to the cytoplasm of the cell where it is ubiquitinated and degraded (Fomenkov et al., 2004).

A schematic representation of the information reviewed in this paragraph (14-3-3 proteins and the p53 family) is shown in figure 3. It must be noted however that the actions described might be cell-type specific or limited to one or a few specific splice variants. Conversely, possibly actions have only been described for one or a few p53 family members or 14-3-3 isoforms while other family members behave in the same or similar way.

P53 family and keratinocytes

The p53 family is undoubtedly involved in the regulation of epidermal keratinocytes and p63 is clearly the most important family member of the three. P63 is absolutely essential for keratinocyte differentiation, as mentioned before, mice lacking p63 are completely unable to form stratified epithelia including epidermis (figure 4).

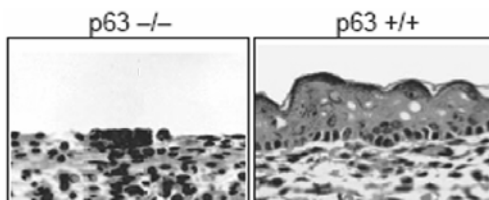


Figure 4. Defects in epidermal differentiation in p63-deficient mice. P63 $-/-$ mice lacking squamous stratification in the epidermis. Picture adapted from Yang et al., 1999.

Keratinocytes arise from epidermal stem cells, which give rise to daughter stem cells and transit amplifying cells through asymmetric cell division. Transit amplifying cells withdraw from the cell-cycle after a few rounds of division and then undergo terminal differentiation (Roop, 1995). At embryonic day 9.5, when the epidermis is still single-layered, commitment to differentiation (stratification) is marked by the expression of keratins 5 and 14 (Byrne et al., 1994). P63 expression can be detected one day before the onset of keratin 14 expression, at embryonic day 8.5, and it is now believed to be the master molecular switch for the induction of stratification (Yang et al., 1998; Koster et al., 2002). TAp63 isoforms are expressed just prior to stratification and Δ Np63 isoforms are the first forms detected after cells have committed to stratification, but before the onset of terminal differentiation (Koster et al., 2004). In mature epidermis, the predominantly expressed p63 form is Δ Np63 α (although other forms are also expressed), which is expressed mainly in the basal layer, whereas all p63 forms are downregulated in the differentiated layers (Yang et al., 1998; Koster et al., 2002; Parsa et al., 1999). The decrease of Δ Np63 α during epidermal differentiation may be required to prevent binding of Δ Np63 α to the 14-3-3 σ promoter and inhibit its expression. Downregulation would allow expression of 14-3-3 σ , which (as previously described in chapter 1) is essential for terminal differentiation of keratinocytes (Koster et al., 2004; Dellambra et al., 2000).

The roles for the other two p53 family-members in keratinocytes are less obvious, mouse knock-out models for p73 (Yang et al., 2000) and p53 (Donehower et al., 1992; Jacks et al., 1994) show no obvious epidermal defects as observed in p63-deficient mice. Nevertheless, indications have been found that p53 and p73 are also involved in keratinocyte differentiation. One clear indication that p73 is involved in keratinocyte differentiation is that p73 γ and δ induce expression of loricrin and involucrin, two markers of epidermal differentiation (De Laurenzi & Melino., 2000). P53 has been implicated in differentiation of keratinocytes because levels of p53 protein decrease during epidermal cell differentiation. The differentiation specific decrease of p53 was accompanied with an elevated transcriptional activity of p53 (Weinberg et al., 1995). Also, p53 overexpression in normal keratinocytes induced expression of the terminal differentiation marker involucrin (Woodworth et al., 1993).

and ectopic expression of p53 into skin cancer cells enhanced expression of proteins required for keratinization (brenner, 1993). In retrospective, however, one should be careful with interpretations like these, as results were obtained at a time when the existence of the related p63 and p73 genes was not yet suspected. However, also after the discovery of p63 and p73, p53 has also been shown to contribute to keratinocyte differentiation as p53 rescued HaCaT cells (non-tumor immortal human keratinocytes) differentiate faster than the parental or control cells (Paramio et al., 2000).

P53 and p73 variants might be involved in regulation of keratinocyte cell fate by regulating the expression of p63 regulated genes. As described previously, p53, p63 and p73 proteins have common targets and therefore targets involved in differentiation transactivated by p63 might be transactivated by p53 or p73 variants as well. Similarly, p53 and p73 variants increase transcription of p63 targets by co-operating with p63 (Flores et al., 2002).

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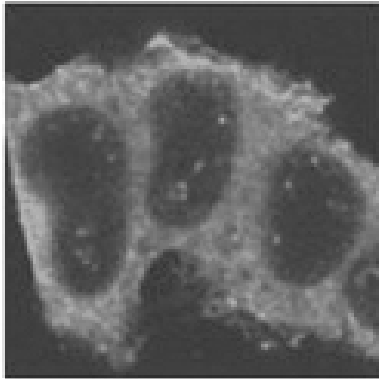
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Chapter 3



Cellular functions of 14-3-3 ζ in apoptosis and cell adhesion emphasize its oncogenic character

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Abstract

14-3-3 proteins are relevant to cancer biology as they are key regulators of major cellular processes such as proliferation, differentiation, senescence and apoptosis. So far, the sigma isoform (14-3-3 σ) has most directly been implicated in carcinogenesis and was recognised as a tumour-suppressor gene. The other six members of the mammalian 14-3-3 gene family likely behave as oncogenes, although direct evidence supporting this view is largely circumstantial. In this report we show that knockdown of 14-3-3 ζ induces at least two isoform-specific phenotypes that are consistent with a potential oncogenic activity during tumorigenesis. First, downregulation of 14-3-3 ζ sensitised cells to stress induced apoptosis and JNK/p38 signalling and second, it enforced cell-cell contacts and expression of adhesion proteins. Apparently, the zeta isoform restrains both cell adhesion and the cellular propensity for apoptosis, two activities that are also restrained during carcinogenesis. The assumption that 14-3-3 ζ has oncogenic properties was substantiated with a web-based meta-analysis (Oncomine), revealing that 14-3-3 ζ is overexpressed in various types of carcinomas. As the highly-conserved human 14-3-3 gene family encodes proteins with either tumour promoting or tumour suppressing activities, we infer that the cellular balance between the various 14-3-3 isoforms is crucial for the proper functioning of cells.

Running title: Oncogenic properties of 14-3-3 ζ

Key words: 14-3-3 zeta isoform; siRNA; UV irradiation; c-Jun N-terminal kinase; p38-MAPK; E-cadherin; Oncomine.

Main text

Of the 7 highly homologous members of the human 14-3-3 gene family, the 14-3-3σ isoform has directly been linked to human cancer. 14-3-3σ is the only family member transactivated by the p53 tumour suppressor in response to DNA damage and as such essential for controlling cell cycle progression in G2/M (Hermeking et al., 1997). Moreover, epigenetic silencing of 14-3-3σ has been found at high frequency in epithelial cancers, indicating that loss of 14-3-3σ expression is crucial to tumour development (Hermeking, 2003). Evidence for a direct role of the other six 14-3-3 isoforms in tumorigenesis is largely circumstantial; intuitively these isoforms are likely to function as potential oncogenes, but activities have also been described that suggest a tumour-suppressing function (Tzivion et al., 2006).

To investigate whether 14-3-3 family members other than sigma have isoform-specific functions relevant for tumorigenesis, we created human HaCaT cell lines stably expressing small interfering RNA (siRNA) targeted specifically to the 14-3-3ζ isoform. Two independent clones, HZ3 and HZ4, showed very low 14-3-3ζ protein levels as compared to two control cell lines HP2 and HP3 (Fig. 1a). No difference was observed with an antibody detecting all human 14-3-3 isoforms, indicating that downregulation of 14-3-3ζ does not significantly influence total cellular 14-3-3 protein levels. Also, the expression of the 14-3-3σ isoform was similar in all cell lines (Fig. 1a). 14-3-3 expression levels were further investigated by quantitative RT-PCR (Fig. 1b). As expected, the HZ3 and HZ4 cell lines showed significantly lower 14-3-3ζ levels than HP2 and HP3 cell lines, whereas RNA levels of 14-3-3θ, 14-3-3ε and 14-3-3β were similar in all four cell lines and 14-3-3γ and 14-3-3η RNA levels were hardly detectable (Fig.1b). These results demonstrate that the 14-3-3ζ isoform was selectively downregulated in the HZ3 and HZ4 cell lines.

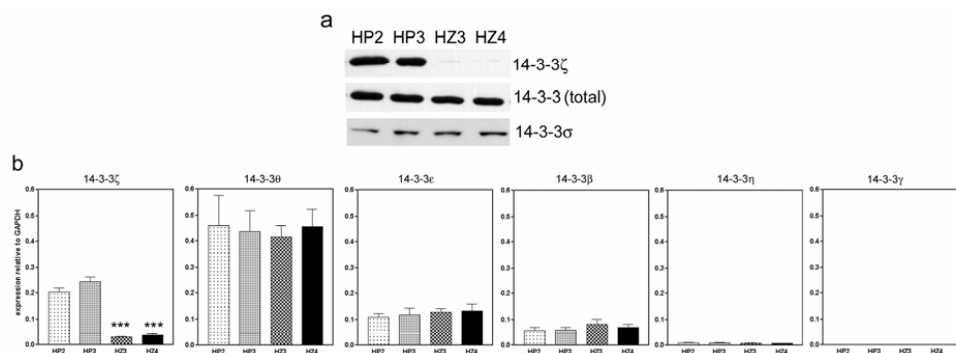


Figure 1. Specific cellular downregulation of 14-3-3 ζ . In all experiments human HaCaT cells were used. These stable cell lines are non-tumorigenic, form a normal differentiated epithelium when transplanted onto nude mice (Boukamp et al., 1988) and have been successfully used for studying early stages of carcinogenesis (Burnworth et al., 2006). Cell lines expressing siRNA specific for 14-3-3 ζ were created by using the target sequence 5'-ACAGCAGATGGCTCGAGAA cloned in pSuper as described (Brummelkamp et al., 2002). Individual puromycin-resistant transfectants were isolated after cotransfection with pPur18, a derivative of pRSV-H20 carrying the puromycin-N-acetyltransferase gene. **a**) 14-3-3 ζ downregulated cells HZ3 & HZ4 and control cells HP2 & HP3 were grown to 80% confluence in DMEM, 10% fetal bovine serum, 1 μ g/ml puromycin at 37°C and 5% CO₂ and lysed directly on the dishes. Protein extracts and Western blotting were as described (Niemantsverdriet et al., 2005). Blots were reacted with 14-3-3 ζ (C-16), 14-3-3 β (K-19) (detects total 14-3-3), 14-3-3 σ (N-14) specific antibodies (Santa Cruz Biotechnology) and detected with either donkey anti-Mouse- or anti-Goat-IgG-HRP and the ECL system (Amersham). **b**) HP2, HP3, HZ3 and HZ4 cells were grown to 80% confluence, RNA was isolated and reverse transcribed as described (Niemantsverdriet et al., 2005). Expression levels of 14-3-3 ζ , 14-3-3 θ , 14-3-3 ϵ , 14-3-3 β , 14-3-3 γ and 14-3-3 η relative to GAPDH (internal control) were determined by Real-time RT-PCR analysis; *** indicates p-values < 0.001 in a two-tailed t-test; error bars represent standard error. (See supplementary methods for more details).

While the spontaneous apoptosis rate was similar in all cell lines, UV-C radiation (10 J/m²) dramatically increased the apoptosis rate in 14-3-3 ζ downregulated cells (8 fold difference with control cells, Fig. 2a). The increased sensitivity for apoptosis of

14-3-3 ζ downregulated cells was paralleled by a significantly higher UV induced activation of p38 (4 fold difference with HP cells) and JNK (2 fold difference) (Fig. 2b). Both p38 and JNK are well known transducers of apoptotic signals (Dent et al., 2003) and were shown to activate the pro-apoptotic protein Bax by inducing its translocation to mitochondria (Kim et al., 2006).

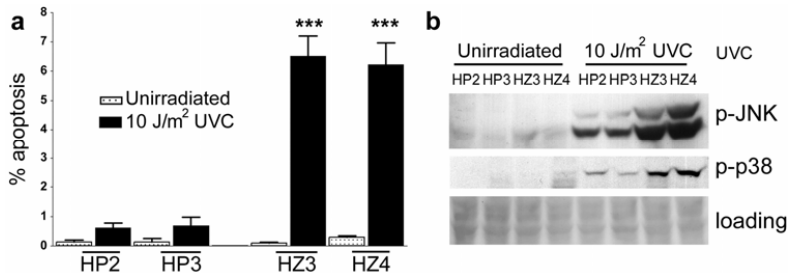


Figure 2. Downregulation of 14-3-3 ζ sensitises cells to UV induced apoptosis. HP2, HP3, HZ3 and HZ4 cells were grown to 80% confluence and either left unirradiated or irradiated with 10 J/m² UV-C. Apoptotic cells were detected by Alexa488-labelled annexin V (Molecular Probes) combined with Hoechst 33258 (Sigma) DNA staining. Images of the same microscopic field were acquired with a Zeiss Axiovert 135 microscope using a phase contrast filter to detect all cells and a DAPI/GFP filter (Zeiss) to detect apoptotic cells. **a**) Graphic representation of the apoptosis percentage of HP2, HP3 and 14-3-3 ζ downregulated HZ3 and HZ4 cells, 24 hours after either mock-irradiation (dotted bars) or irradiation with 10 J/m² UV-C (black bars). *** indicates p-values < 0.001 in a two-tailed t-test ; error bars represent standard error. **b**) Immunoblot analysis of untreated or irradiated (10 J/m² UV-C) HP2, HP3, HZ3 and HZ4 cells with phosphospecific antibodies detecting respectively activated JNK (p-JNK) (lower band: JNK-1; upper band: JNK-2) and activated p38 (p-p38) (Cell Signalling Technology). Cell extracts were prepared 1 h after UV irradiation, and detection was as described in figure 1. Ponceau S staining of the blot confirms equal loading.

This apoptotic pathway might actually be potentiated by release of phosphorylated BAD from 14-3-3 ζ sequestration, resulting in mitochondrial re-localization and a concomitant neutralization of the anti-apoptotic function of Bcl-X_L (Hekman et al., 2006). No constitutive p38 or JNK phosphorylation was observed in

any of the 4 cell lines. It is also interesting to mention that the mitogenic ERK pathway (assessed following serum stimulation) was not affected by 14-3-3 ζ downregulation (supplementary figure S1). Summarizing, our data demonstrate that in normal cells 14-3-3 ζ inhibits specifically stress induced p38 and JNK signalling and the triggering of the apoptotic response. Effective apoptosis is essential to prevent tumorigenesis in humans and evasion of apoptosis is considered one of the typical hallmarks of cancer cells (Hanahan and Weinberg, 2000).

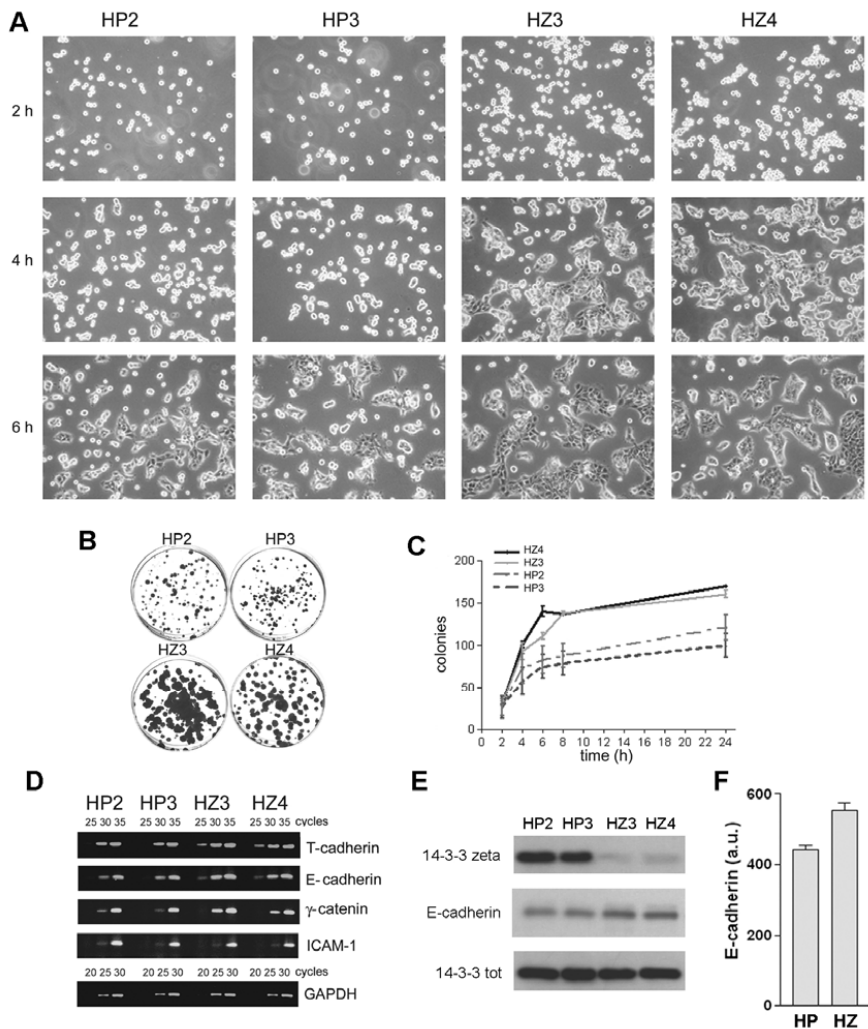


Figure 3. see next page for legend.

Figure 3. Downregulation of 14-3-3ζ results in enhanced cell adhesion. **A)** Control cells HP2/HP3 and 14-3-3ζ downregulated HZ3/HZ4 cells were grown to 80% confluence, trypsinized, pipetted vigorously up and down (at least 10x) to prevent clustering in suspension, seeded (10^6 cells per dish) for the indicated time and photographed under phase contrast illumination. **B)** HP2, HP3, HZ3 and HZ4 cells were treated as above, seeded onto 6 cm dishes at 1000 cells per 5 ml medium containing 1 µg/ml puromycin and incubated for 2 weeks under standard cell culture conditions. Cell colonies were visualized with Coomassie Brilliant Blue (CBB) staining. **C)** Variation of the total number of HP2, HP3, HZ3 and HZ4 colonies relative to the time allowed for plating. Cells were isolated, processed as above, seeded for the indicated time period, supplied with fresh medium and incubated for 10 days before colonies could be detected by CBB staining and counted. Bars represent standard error. **D)** Semi-quantitative RT-PCR analysis of T-cadherin, E-cadherin, γ-catenin, ICAM-1 and GAPDH (control) expression at the indicated number of PCR cycles. Note the difference in band intensity between 14-3-3ζ downregulated (HZ3/HZ4) and control cells (HP2/HP3) after 25 PCR cycles for T-cadherin and E-cadherin and at 30 cycles for γ-catenin (see supplementary information for more details). **E)** Western blot showing up-regulation of E-cadherin protein in 14-3-3ζ downregulated cells (HZ3/HZ4). Total 14-3-3 and 14-3-3ζ are included as internal controls (see also Fig. 1). 14-3-3 antibodies were as documented in the legend of Fig. 1. For E-cadherin detection a monoclonal antibody from BD Transduction Laboratories (Cat. #610181) was used. **F)** Quantification of E-cadherin expression levels in Fig. 3E., indicating a significant increase in E-cadherin proteins in 14-3-3ζ downregulated cells (HZ) versus control cells (HP).

During culturing of the cell lines, we noticed differences in plating between 14-3-3ζ down-regulated and control cells. Control cells attached to the culture dish as single cells or clusters of only a few cells, while the 14-3-3ζ downregulated cells appeared to have stronger cell-cell contacts as they clustered in larger groups (Fig. 3A, 2 hr). In a colony formation assay, 14-3-3ζ downregulated cells showed significantly larger colonies than control cells, although all four cell lines had equal growth rates (supplementary figure S1), an observation which corroborates with our finding that mitogenic ERK signalling is not affected by 14-3-3ζ downregulation. Apparently, due to enhanced cell-cell contacts, resulting in early clustering, 14-3-3ζ

downregulated colonies are initiated by multiple cells in contrast to the control colonies (Fig. 3B). Additionally, 14-3-3 ζ downregulated cells attached to the culture dish much faster as judged from the flat appearance that was visible already 4 hours after seeding, a time point where control cells had still a globular shape (Fig. 3A, middle panel). The graphical analysis in Fig. 3C shows that the resulting difference in total number of colonies between HZ and HP cells are statistically significant and due to early time points of plating. In order to investigate whether these differences depend on active processes involving cell adhesion proteins (Balda and Matter, 2003), we compared the expression levels of intracellular cell adhesion molecule 1 (ICAM-1), T-cadherin, E-cadherin and γ -catenin between control and 14-3-3 ζ downregulated cells by RT-PCR analysis. While RNA levels of ICAM-1 were similar in all cell lines, RNA levels of adhesion proteins T-cadherin, E-cadherin and γ -catenin were significantly higher in 14-3-3 ζ downregulated cells (Fig. 3d).

Increased transcription of E-cadherin in these cells was also reflected by significantly (approximately 25%) higher protein levels (Fig. 3E, F). Tissue invasion and metastasis, like evasion of apoptosis, represents one of the six hallmarks of cancer and is directly linked to loss of adhesion proteins (Hanahan and Weinberg, 2000). Loss of E-cadherin correlates with the invasiveness of human epithelial cancers. It has been documented for tumours from various organs and is considered a significant prognostic marker (Beavon, 2000). Downregulation of T-cadherin is believed to contribute to tumour invasiveness in basal cell carcinomas (Takeuchi et al., 2002). Similarly, cancer patients with loss of γ -catenin expression are considered a high-risk group for tumour metastasis and poor prognosis (Clairotte et al., 2006; Ueda et al., 2006).

Taken together, the results described above indicate that the 14-3-3 ζ isoform restrains two cellular processes, stress-induced apoptosis and cell adhesion, which are also suppressed during carcinogenesis. Hence, we inferred that high expression of 14-3-3 ζ might contribute to tumorigenesis. In order to find support for this hypothesis, we performed a web-based meta-analysis using the ONCOMINE platform, that allows a direct comparison of gene expression profiles in tumours versus the corresponding normal tissues (Rhodes and Chinnaiyan, 2005; Rhodes et al., 2004).

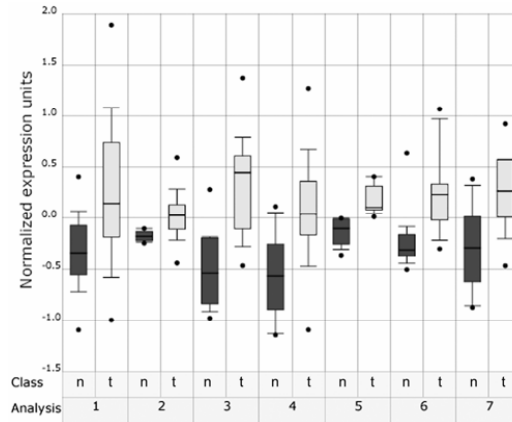


Figure 4. Overexpression of 14-3-3ζ in tumours (t = light bars) as compared to the corresponding normal tissue (n = dark bars). Data analysis was performed with the ONCOMINE 3.0 database (Rhodes and Chinnaiyan, 2005; Rhodes et al., 2004) using standard settings (www.oncomine.org). The following comparisons were performed: analysis 1) non-tumour liver vs hepatocellular carcinoma, 2) non-neoplastic lung vs lung adenocarcinoma, 3) normal testis vs seminoma, 4) normal lung vs lung adenocarcinoma, 5) normal pancreas vs pancreatic adenocarcinoma, 6) normal lung vs carcinoid, 7) normal lung vs squamous cell lung carcinoma.

In this analysis, 14-3-3ζ was clearly up-regulated in human hepatocellular carcinoma, seminoma, pancreatic adenocarcinoma and several types of lung carcinoma (Fig. 4). Similarly, studies on human stomach cancer (Jang et al., 2004), oral squamous cell carcinoma (Arora et al., 2005) and breast cancer (Zang et al., 2004), have identified 14-3-3ζ among the most significantly increased proteins/genes in the tumours as compared to the corresponding normal tissues. Furthermore, 14-3-3ζ maps to human chromosome 8q23, a region frequently amplified in metastatic cancer (Ghadimi et al., 2003; Tada et al., 2000).

As 14-3-3 proteins are highly homologous, have similar protein structures, bind to several binding partners with similar affinities, the most common view so far has been that the various isoforms influence particular cellular processes in an analogous way (van Hemert et al., 2001). However, the work presented here and

recent work by others indicate that individual 14-3-3 proteins possess isoform-specific functions and might selectively affect distinct aspects of the carcinogenic process. Whereas 14-3-3 σ is a downstream-target of p53 (Hermeking, 2003) and 14-3-3 γ is an upstream-regulator of p53 (Jin et al., 2006), both isoforms promote DNA damage induced cell cycle arrest. On the contrary, overexpression of the 14-3-3 η isoform counteracts the same process (Wanzel et al., 2005). The 14-3-3 τ isoform stimulates apoptosis by stabilising E2F1 (Wang et al., 2004), whereas the data presented here reveal an inhibitory activity of 14-3-3 ζ on UV-induced apoptosis.

The cellular phenotypic changes that we have registered are the result of downregulation of one specific isoform, without significantly changing the total cellular 14-3-3 content. Hence, we infer that the intracellular balance between 14-3-3 isoforms is crucial for the proper functioning of cells. As mentioned before, the 14-3-3 σ is silenced in many types of cancer and is considered a tumour-suppressor (Hermeking, 2003). Downregulation of 14-3-3 η was found in glioblastoma and this isoform might also act as tumour-suppressor (Buckley et al., 2002). In sharp contrast, the data presented here reveal that 14-3-3 ζ is up-regulated in cancer and likely behaves as an oncogene. Studies in rodents strongly indicate that also 14-3-3 β has oncogenic properties (Sugiyama et al., 2003; Takihara et al., 2000). In order to fully understand how deregulation of the cellular 14-3-3 balance can contribute to tumour development, it will be imperative to delineate the precise contribution of each 14-3-3 isoform in this process. From the results presented here we anticipate that deregulation of the 14-3-3 balance by overexpression of 14-3-3 ζ contributes to tumorigenesis by inhibition of apoptosis and cell adhesion.

14-3-3 proteins are potential targets for cancer therapeutics (Porter et al., 2006) and it has been proposed that targeting the 14-3-3 family as a whole might reduce the development of resistant tumour cells (Hermeking, 2003). However, if deregulation of the intracellular balance between the various isoforms, rather than changes of the total cellular 14-3-3 content, is important for tumorigenesis, targeting specific isoforms might be more valuable in anticancer therapy. In line with the view

presented in this study, preliminary data mentioned by Tzivion *et al* (Tzivion et al., 2006) indicate that a slight increase of 14-3-3 ζ is sufficient to induce tumorigenesis in mice. Hence, when targeting 14-3-3 for therapeutic purposes, the 14-3-3 ζ isoform might be the target of choice in the future.

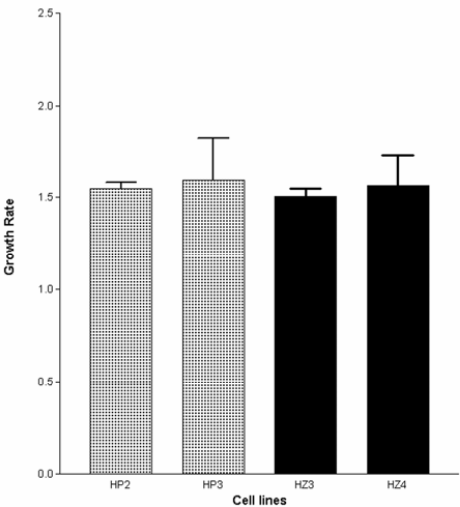
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Supplementary information

Supplementary figure S1:

A.



B.

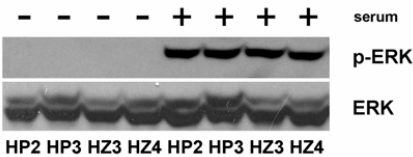


Figure S1. A. Comparison of growth rates between HP2, HP3, HZ3 and HZ4. Cells were seeded in two 24-wells plates, 10^4 cells/well, 4 wells/cell line and incubated at 5% CO_2 at 37°C in standard medium containing 1 $\mu\text{g}/\text{ml}$ puromycin. 3 (plate 1) and 4 (plate 2) days after seeding, growth medium was replaced with growth medium containing 50 $\mu\text{g}/\text{ml}$ WST-1 (Roche Applied Science) and cells were further incubated for exactly 4 hours. Wst-1 reduction was measured in a Tecan GENios plate reader at 420nm. Growth rates are defined as the increase in relative cell number between two consecutive days (24 h) and was calculated by dividing the average OD_{420} of a cell line measured at day 4 by the average OD_{420} of the same cell line measured at day 3. Similar results were obtained at earlier consecutive days.

B. Downregulation of 14-3-3 zeta does not influence mitogenic signalling cascades. Cells were grown until 50% confluence, serum starved for 12 hours and treated with medium with serum for 30 min (+ serum) or left for the same time in DMEM without serum (- serum) before extracts were prepared and analyzed with phospho-ERK (p-ERK) or ERK specific antibodies. Detection was as described in the legend of Fig. 1.

Supplementary Methods:

RT-PCR

RNA isolation and RT-reaction were performed essentially as described previously (Niemantsverdriet et al., 2005). Quantitative Real-Time PCR was performed in a Biorad iCycler in 25µl, using 10 pmol of each primer (see below), SuperTaq+ (HT Biotechnologies), SYBR® Green I (Molecular Probes), and Fluorescein (Biorad); results were analysed with the iCycler program (Biorad) using dilution series with known concentrations of purified target sequence PCR product to calculate the molar expression levels. For semi-quantitative RT-PCR, PCR was performed in 50µl for the indicated amount of cycles, using 20 pmol of gene-specific primers, 1.5mM MgCl₂ and 1.25 Units of Amplitaq GoldTM (Roche). Expression levels of 14-3-3 RNA were standardised using the expression level of GAPDH. The various PCR primers that were used are represented in table 1.

Table 1: Used PCR primers

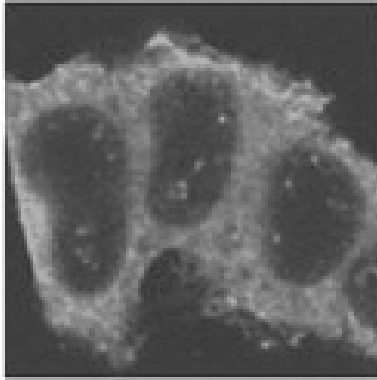
gene	primer 1	primer 2
GAPDH	5'- CGGAGTCAACGGATTTGGTCGTAT	5'- AGCCTTCTCCATGGTGGTGAAGAC
14-3-3ζ	5'- GCAAGGAGCTGAATTATCCAATG	5'- GTTGTGACTGATCGACAATCCC
14-3-3θ	5'- AGCAGAAGACCGACACCTCC	5'- CCGTGGTGAGATGGATC
14-3-3ε	5'- GAAGAAAACAAGGGAGGAGAAGAC	5'- CTCCGCAGCCTCCTTC
14-3-3β	5'- GCAGTCACAGAACAGGGGC	5'- CTATCTTCTCACGGTACTCTTTGC
14-3-3η	5'- GTGACAGAGCTGAATGAACCTCTC	5'- CCAATTTCTTTTCGTTTCCATC
γ-catenin	5'- ATGGAGGTGATGAACCTGATGG	5'- CGTTTGGCCCTGGCTG
T-cadherin	5'- GCCGGTGCCTCTGGAAG	5'- GGCTGTGGCCGTGCCTG
E-cadherin	5'- TCCATTTCTTGGTCTACGCC	5'- GCCAGGAGAGGAGTTGGG
ICAM-1	5'- GAGGCTCCGTGCTGGTGAC	5'- CGGTGAGGAAGGTTTTAGCTGTTG

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Chapter 4



Isoform-specific differences in rapid nucleocytoplasmic shuttling cause distinct subcellular distributions of 14-3-3 σ and 14-3-3 ζ

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Abstract

Nucleocytoplasmic transport of proteins plays an important role in the regulation of many cellular processes. Differences in nucleocytoplasmic shuttling can provide a basis for isoform-specific biological functions for members of multigene families, like the 14-3-3 protein family. Many organisms contain multiple 14-3-3 isoforms, which play a role in numerous processes, including signalling, cell cycle control and apoptosis. It is still unclear whether these isoforms have specialised biological functions and whether this specialisation is based on isoform-specific ligand binding, expression regulation or specific localisation. Therefore, we studied the subcellular distribution of 14-3-3 σ and 14-3-3 ζ in vivo in various mammalian cell types using yellow fluorescent protein fusions and isoform-specific antibodies. 14-3-3 σ was mainly localised in the cytoplasm and only low levels were present in the nucleus, whereas 14-3-3 ζ was found at relatively higher levels in the nucleus. Fluorescence recovery after photobleaching (FRAP) experiments indicated that the 14-3-3 proteins rapidly shuttle in and out of the nucleus through active transport and that the distinct subcellular distributions of 14-3-3 σ and 14-3-3 ζ are caused by differences in nuclear export. 14-3-3 σ had a 1.7x higher nuclear export rate constant than 14-3-3 ζ , while import rate constants were equal. The 14-3-3 proteins are exported from the nucleus at least in part by a Crm1-dependent, leptomycin B-sensitive mechanism. The differences in subcellular distribution of 14-3-3 that we found in this study are likely to reflect a molecular basis for isoform-specific biological specialisation.

Key words: 14-3-3 Proteins, 14-3-3 Localisation, Nucleocytoplasmic transport, Fluorescence recovery after photobleaching, FRAP

Introduction

The 14-3-3 proteins form a family of highly conserved acidic dimeric proteins, present in all eukaryotic organisms studied so far (for recent reviews, see Yaffe, 2002; Tzivion and Avruch, 2002; van Hemert et al., 2001). They are able to associate with over 100 binding partners and are involved in the regulation of a wide range of cellular processes, including signalling, cell cycle control, apoptosis, exocytosis, cytoskeletal rearrangements, transcription and enzyme activity.

Many organisms contain multiple isoforms – for example, in mammals nine isoforms have been identified (α , β , γ , δ , ϵ , ζ , η and σ), but the reason for this variety remains unclear. It is not yet understood whether isoforms have distinct and specialised functions or whether they are just under the control of temporal and tissue specific regulation. Variations in temporal, developmental and tissue-specific isoform expression have indeed been reported (Yaffe, 2002; Leffers et al., 1993; Rittinger et al., 1999; Roth et al., 1994; Perego and Berruti, 1997; Tien et al., 1999). In addition, the expression of several plant and mammalian isoforms is influenced by external stimuli like temperature, injury and different types of stress (Yaffe, 2002; Jarillo et al., 1994; de Vetten and Ferl, 1995; Chen et al., 1994; Kidou et al., 1993). Besides differences in expression patterns, there are several reports on isoform-specific interactions with 14-3-3 binding partners (Yaffe, 2002; Wakui et al., 1997; Craparo et al., 1997; Meller et al., 1996; Liu et al., 1997; Van Der Hoeven et al., 2000; Tang et al., 1998; Peng et al., 1997; Zhang et al., 1997; Kumagai et al., 1998; Kurz et al., 2000; Hashiguchi et al., 2000). However, many functions can be performed by all 14-3-3 isoforms. Several binding partners, like Raf-1, BAD and Cbl, associate with all isoforms (Yaffe, 2002; Rittinger et al., 1999; Subramanian et al., 2001), and several 14-3-3-associating peptides have similar affinities for all isoforms (Muslin et al., 1996; Petosa et al., 1998). It is therefore likely that a combination of isoform-specific expression, localisation and functional specialisation is the basis for the existence of multiple isoforms.

In this report we focus on the human σ and ζ isoforms. 14-3-3 ζ is abundant in various tissues and has been reported to interact with many of the known 14-3-3 ligands. 14-3-3 σ (also called stratifin) is mainly expressed in epithelial cells (Leffers

et al., 1993) and is strongly induced by ionising radiation and DNA damage through a p53-responsive promoter element (Hermeking et al., 1997). In addition, it is involved in differentiation of keratinocyte stem cells (Pellegrini et al., 2001). 14-3-3 σ appears to be involved in oncogenesis as it is downregulated in many breast cancers (reviewed by Yaffe, 2002) and hepatocellular carcinoma cells (Iwata et al., 2000), and it is overexpressed in several other cancers (Yaffe, 2002). 14-3-3 σ acts as a tumor suppressor, inducing a G1 arrest by inhibiting Cdk2 and causing a G2 arrest by sequestering cyclin B1-cdc2 in the cytoplasm (Laronga et al., 2000; Chan et al., 1999; Hermeking et al., 1997). In contrast to 14-3-3 ζ and other isoforms, 14-3-3 σ does not bind to CDC25 (Peng et al., 1997).

Several 14-3-3 isoforms have been reported to be abundant in the cytoplasm and to be present in the nucleus of various organisms (Todd et al., 1998; Markiewicz et al., 1996; Fanger et al., 1998; Kumagai and Dunphy, 1999; Dalal et al., 1999; van Zeijl et al., 2000). 14-3-3s were also found to be associated with membranes (Martin et al., 1994; Roth et al., 1994; Jones et al., 1995; Tien et al., 1999), the cytoskeleton (Garcia et al., 1999; Ku et al., 1998; Roth and Burgoyne, 1995; Roth et al., 1994) and centrosomes (Pietromonaco et al., 1996). Some reports suggested a specific subcellular distribution for certain isoforms (Roth et al., 1994; Martin et al., 1994; Sehnke et al., 2000; Pietromonaco et al., 1996). However, a detailed comparison of isoform-specific localisation in one system has not been performed and all published studies are based on immunolocalisations in fixed cells. Here, we report on the isoform-specific and dynamic localisation of 14-3-3 σ and 14-3-3 ζ in living mammalian cells. We used yellow fluorescent protein (YFP) fusion constructs and showed that these were functional *in vivo* in yeast. We found that 14-3-3 σ had a mainly diffuse cytoplasmic distribution in various mammalian cell types and that only low levels were present in the nucleus. By contrast, 14-3-3 ζ was present at higher levels in the nucleus. We showed that both 14-3-3 proteins rapidly shuttle in and out of the nucleus, in part via a Crm1-mediated nuclear export mechanism. We performed fluorescence recovery after photobleaching (FRAP) experiments on living cells from which we conclude that a difference in nuclear export causes the different subcellular distributions of 14-3-3 σ and 14-3-3 ζ .

Materials and Methods

Strains, media and cell culture

The *Saccharomyces cerevisiae* strains used in this study were derived from GG1306 (MATa leu2,3-112 ura3-52 trp1-92 his4 bmh1::LEU2 bmh2::APT1 Ycplac33[BMH2] (van Heusden et al., 1995)). Yeast strains were grown at 30°C in either MY or MYZ with 1% galactose (Zonneveld, 1986), supplemented as required with 30 µg/ml uracil, 20 µg/ml tryptophan, 20 µg/ml histidine and 1 mg/ml 5-fluoroorotic acid (5-FOA). Yeast was transformed according to the method of Gietz et al. (Gietz et al., 1995). NHK cells (normal human keratinocytes) were grown as described previously (Sark et al., 1998). Hela, HaCaT, NIH-3T3 and NHF cells (normal human fibroblasts, strain F9) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F10, supplemented with 10% fetal bovine serum (Hyclone), streptomycin and penicillin.

Plasmids

The sequences of the oligonucleotides used in this study are listed in Table 1.

Table 1. Oligonucleotide sequences

P1	5'-CCGATGGATCCATGGAGAGAGCCAGTCTGATC - 3'
P2	5'-CTATGGATCCCCGCCACCGCTCTGGGGCTCCTGGGGAG - 3'
P3	5'-CGCGCGGATCCATGGATAAAAATGAGCTGGTTC - 3'
P4	5'-CGCGGATCCCCGCCACCATTTTCCCTCCTTCTCCTG - 3'
P5	5'-AGCTGAATTCCTCGAGATGAGAGTTCTCATCACCATCACCATCACGGGAATTCCTGGCTAGAAGCTTTA AGCGGCCGC - 3'
P6	5'-CTAGGCGGCCGCTTAAAGCTTCTAGCCCGGAATTCCTCGTATGGTGATGGTGATGAGAACCTCTCATCT CGAGGAATTC - 3'
P7	5'-AATTAATAAAAAAATATGGAGAGAGCCAGTCTGATCCAGAAGCCAAGCTGGCAGAGCAGGCCGAACG CTATGAGGACATGGCAGCCTTCATGAAAGG - 3'
P8	5'-CGCCTTTCATGAAGGCTGCCATGTCCTCATAGCGTTCGCGCTGCTCTGCCAGCTTGGCCTTCTGGATCAGAC TGGCTCTCTCCATTATTTTTTATT - 3'
P9	5'-GGGGTACAAAAAATGGATAAAAATGAGCTGGTTC - 3'
P10	5'-GCTCTAGATTAATTTTCCCTCCTTCTCC - 3'
P11	5'-AATTAGATCTAAAAAATGCGAGGTTCTCATCACCATCACCATCACGG - 3'
P12	5'-CCGTATGGTGATGGTGATGAGAACCTCGCATTATTTTAGATCT - 3'
P13	5'-AGCTGAATTCCTGGGCTAGAAGCTTTAAGCGGCCGC - 3'
P14	5'-CTAGGCGGCCGCTTAAAGCTTCTAGCCCGGAATTC - 3'

Plasmids pMP4553 and pMP4558 encode 14-3-3 σ and 14-3-3 ζ , respectively, tagged C-terminally with YFP and under the control of the CMV promoter. pMP4552 and pMP4553 were constructed by cloning a fragment generated by PCR with primers P1 and P2 on a human keratinocyte cDNA library (Marenholz et al., 2001), followed by digestion with BamHI, in pECFP-N1 and pEYFP-N1 (Clontech), respectively. A similar strategy was used to construct pMP4557 and pMP4558, which encode 14-3-3 ζ , C-terminally tagged with CFP and YFP, respectively. For the latter constructs, a fragment generated by PCR with primers P3 and P4 on I.M.A.G.E. Consortium (LLNL) cDNA Clone 531246 (Lennon et al., 1996), followed by digestion with BamHI, was used as insert. pYES-TRP[BMH2] allows the galactose inducible expression of BMH2 (van Heusden et al., 1996). Yeast expression vector pMP4550, containing the TRP1 marker and the GAL1 promoter, was constructed by replacing the URA3 gene of pYES2 (Invitrogen) with a 1.8 kb TRP1 containing ClaI-BglI fragment from YEplac112 (Gietz and Sugino, 1988). Plasmid pMP4570 enables the galactose inducible expression of 14-3-3 σ . This plasmid was made by inserting a synthetic DNA fragment consisting of oligonucleotides P5 and P6 in the HindIII-XbaI sites of pMP4550, followed by cloning a 0.8 kb 14-3-3 σ containing SalI-BsrBI fragment from pMP4552 in the XhoI-SmaI sites of the resulting construct. Subsequently, a synthetic DNA fragment consisting of oligonucleotides P7 and P8 was cloned in the EcoRI-NarI sites of this plasmid to improve expression. Plasmid pMP4585 enables the galactose inducible expression of 14-3-3 ζ in yeast. A fragment containing 14-3-3 ζ flanked by KpnI and XbaI sites generated by PCR on pMP4558 with primers P9 and P10 was cloned in the KpnI-XbaI sites of pMP4550. pMP4581 encodes a YFP and 6xHis-tagged 14-3-3 σ under control of the GAL1 promoter and was constructed by cloning a 14-3-3 σ -YFP-containing XmaI-NotI fragment from pMP4553 in pMP4571, after which a synthetic DNA fragment consisting of oligonucleotides P11 and P12 was inserted in the EcoRI-SmaI sites of this plasmid. pMP4583, which encodes 6xHis-14-3-3 ζ -YFP, was made in an analogous way, except that a 1.25 kb 14-3-3 ζ -YFP-containing XmaI-NotI fragment from pMP4558 was used. Replacement of a 1.25 kb XmaI-NotI of pMP4583 with a 14-3-3 ζ -CFP containing XmaI-NotI fragment from pMP4557 yielded plasmid pMP4587. Plasmid pMP4584, which allows the expression of 6xHis-14-3-3 σ -CFP in yeast, was made by inserting a

synthetic DNA fragment consisting of oligonucleotides P13 and P14 in the HindIII-XbaI sites of pMP4550, followed by cloning a 1.4 kb 14-3-3 σ -CFP containing SfiI-NotI fragment from pMP4552 in the SfiI-NotI sites of the resulting plasmid.

Purification and analysis of recombinant 14-3-3 proteins

Yeast strains expressing various human 14-3-3 fusion proteins were grown in MYZ, 1% galactose, uracil, histidine to an OD₆₂₀ of 0.5. All steps were performed at 4°C. Cells were lysed with glass beads in FastProtein Red tubes (Bio101) with a Fastprep FP120 (Bio101) in 50 mM Tris-HCl pH 8, 300 mM NaCl, 1% triton X-100, Mini Complete protease inhibitors, EDTA free (Roche) and 5 mM sodium-orthovanadate. Lysates were cleared by centrifugation and applied to a Ni²⁺-NTA agarose (Qiagen) column. The column was washed with 50 mM Tris-HCl pH 8, 300 mM NaCl, followed by a gradient of 10-50 mM imidazole in the same buffer. Elution was done with 500 mM imidazole in 50 mM Tris-HCl pH 6.5, 300 mM NaCl. The eluted protein was dialysed twice against 500 volumes of 50% glycerol, 50 mM Tris-HCl pH 7.6, 300 mM NaCl, 1 mM DTT. Concentrations were determined by Bradford analysis and purity was estimated by SDS-PAGE followed by Coomassie staining. Gel filtration analysis of purified proteins was done with a 70 ml Sephacryl S200 column (Pharmacia) on a BioLogic system (Biorad), using molecular weight standards (Sigma) for calibration.

Cellular fractionation and western blot analysis

HaCaT cells were fractionated into nuclear extract and cytosol using the method of Brunet *et al.* (Brunet *et al.*, 2002). Five micrograms of total protein from the cytosolic and nuclear extract were separated by 12% SDS-PAGE and proteins were transferred to PVDF by semi-dry blotting in 39 mM glycine, 48 mM Tris, 0.08% SDS, 20% methanol. 14-3-3 σ and 14-3-3 ζ were detected with specific antibodies (N-14 and C-16, respectively) from Santa Cruz Biotechnology. No cross-reactivity was found with these antibodies in a test with purified 14-3-3 proteins. Antibodies against actin (C-2), α -tubulin (B-7), PKC (C-20), RasGAP (B4F8) and p53 (DO-1) (Santa Cruz

Biotechnology) were used in control experiments. All antibodies were diluted 1:1000. The BM Chemiluminescence Western Blotting Kit (Roche-Boehringer) was used for detection. Yeast protein extracts were subjected to immunoblotting as described above, except that Pan 14-3-3 antibodies were used to detect human 14-3-3 isoforms and anti-Bmh1p antiserum (van Heusden et al., 1995) was used to detect yeast 14-3-3 proteins. Antisera were diluted 1:5000.

Localisation of YFP-labelled 14-3-3 isoforms

Various mammalian cells were transfected with pMP4553, pMP4558 or pEYFP-N1 using DOTAP (N-[1-(2,3-dioleoyloxy)]-N,N,N-trimethylammoniumpropane-methylsulfate) (Roche). When desired, 20 ng/ml leptomycin B (LMB) was added to the medium 24 hours after transfection and cells were analysed at 0, 2, 4, 5 and 24 hours after addition with a Leica TCS NT confocal microscope. YFP was excited at 514 nm and emission was detected at 526-595 nm. Nuclear and cytoplasmic 14-3-3 levels were determined by quantifying the average YFP fluorescence of regions in the nucleus and cytoplasm of a z-section through the middle of the nucleus using the ImageJ program. The F_N/F_C ratio was calculated by dividing the nuclear value by the cytoplasmic value.

Immunofluorescence microscopy

HaCaT and Hela cells were grown on glass coverslips, fixed with methanol:acetic acid (3:1) and permeabilized with 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% gelatine, 0.5% Triton X-100. Cells were stained overnight with specific anti-14-3-3 σ (N-14) or anti-14-3-3 ζ (C-16) antibodies (Santa Cruz Biotechnology), diluted 1:10. The primary antibody was omitted in control experiments. After washing three times, the coverslips were incubated three hours with Alexa-568 donkey anti-goat IgG (Molecular Probes) or Alexa-568 goat anti-rabbit IgG (Molecular Probes), diluted 1:50. After washing five times with PBS, cells were analysed with a Leica TCS NT confocal microscope. Alexa-568 was excited at 568 nm and emission was detected at 590-635 nm. Image analysis and quantification were done as described previously for YFP-labelled isoforms.

FRAP analysis

Fluorescence recovery after photobleaching (FRAP) experiments were done on a Leica TCS NT confocal microscope. YFP was excited at 514 nm at a laser power of 0.8 mW and emission was detected at 526-595 nm. After recording a pre-bleach image, a region covering >75% of the nucleus was photobleached by zooming in and scanning at high speed with maximum laser power (8 mW) for 3 seconds. Subsequently, images were captured at 1-second intervals during 2 minutes for YFP-labelled 14-3-3 proteins or at 4-second intervals during 8 minutes for YFP alone. During recording of the pre-bleach and time-lapse images, no significant photobleaching was observed for cells for which the photobleaching step was omitted. The change in nuclear fluorescence signal over time ($F_N(t)$) was quantified by image analysis with the LCS-NT software (Leica). $F_N(t)$ was normalised such that $F_N=0$ corresponds to the level of fluorescence immediately after photobleaching and $F_N=1$ corresponds to the final level of fluorescence at the end of the experiment. From the steady-state intensity distribution of nuclear (F_N) versus cytosolic fluorescence signal (F_C), the ratio of nuclear import (k_i) and nuclear export rate constant (k_e) was calculated using $k_i/k_e = F_N/F_C$. For analysis of the fluorescence recovery, we assumed that the nuclear volume was much smaller than the cytosolic volume, which means that F_C is constant and independent of the photobleaching process (actually we found that F_C decreased by <10%). Furthermore, it was assumed that photobleaching does not influence the import and export rates of 14-3-3. In this case exchange of photobleached molecules is governed by k_e and the process can be described by a first-order rate equation leading to a fluorescence recovery of $F_N(t) = 1 - e^{-k_e t}$. Data were fit to this equation leading to a value for k_e , and in combination with the steady-state distribution to the import rate constant $k_i = k_e \cdot F_N/F_C$. The half time $\tau(1/2)$, the time required to reach 50% recovery, was calculated using $\tau(1/2) = \ln 2 / k_e$. All calculations were based on measurements on at least 15 cells from two independent experiments.

Results

Recombinant YFP- and CFP-labelled human 14-3-3 proteins are functional in yeast

We set out to study the 14-3-3 σ and 14-3-3 ζ proteins in living human cells by making CFP and YFP fusion proteins. To test whether these are functional, we used a yeast-based assay. Disruption of the yeast 14-3-3 genes *BMH1* and *BMH2* is lethal and can be complemented by genes encoding 14-3-3 proteins from other organisms (van Heusden et al., 1995; van Heusden et al., 1996). Yeast strain GG1306 contains a *bmh1 bmh2* disruption, which is complemented by the *BMH2* gene on plasmid YCplac33[*BMH2*]. This strain was transformed with plasmids encoding wild-type and various recombinant 14-3-3 σ or 14-3-3 ζ isoforms under the control of the galactose-inducible *GAL1* promoter (Fig. 1A, left panel). The resulting strains were transferred to minimal medium containing galactose, 5-FOA, uracil and histidine, on which they can only grow when they are able to lose plasmid YCplac33[*BMH2*]. Strains expressing human 6xHis-14-3-3 ζ -CFP or 6xHis-14-3-3 ζ -YFP grow as good as cells expressing the yeast *Bmh2p* protein (Fig. 1A, right panel), indicating that these 14-3-3 isoforms can complement the *bmh1 bmh2* disruption. Human 14-3-3 σ constructs also complement, although strains expressing these proteins grow slower compared with strains expressing *Bmh2p* or 14-3-3 ζ . A strain carrying the empty vector (Fig. 1A, strain 1) was not able to grow on 5-FOA containing medium. To rule out the possibility that mutations in the *URA3* gene of plasmid YCplac33[*BMH2*] enabled strains to grow on medium with 5-FOA, we checked for the absence of plasmid YCplac33[*BMH2*] by PCR. A 400 bp product is formed with plasmid YCplac33[*BMH2*] as template and a 600 bp internal control product is formed with the genomic *bmh2* locus as template. Before 5-FOA treatment, both products were obtained (Fig. 1B, glucose). After 5-FOA treatment the 400 bp product was absent, while the 600 kb control product was still present (Fig. 1B), indicating the absence of YCplac33[*BMH2*]. In addition, the absence of the yeast 14-3-3 proteins in strains growing on 5-FOA was shown by western blotting with an antibody against *Bmh1p* and *Bmh2p* (Fig. 1C). The clear band present in a strain expressing *Bmh2p* (Fig. 1C, strain 2) is

absent in the strains expressing human isoforms. The presence and expression level of recombinant human 14-3-3 isoforms was investigated by western blotting with a Pan 14-3-3 antiserum. The yeast Bmh2 protein was not recognised by this antiserum (Fig. 1D, lane 2). The mobility of 14-3-3 σ , 14-3-3 ζ and the 6xHis-tagged CFP and YFP fusion proteins was in agreement with their predicted mass (Fig. 1D, lane 3-8).

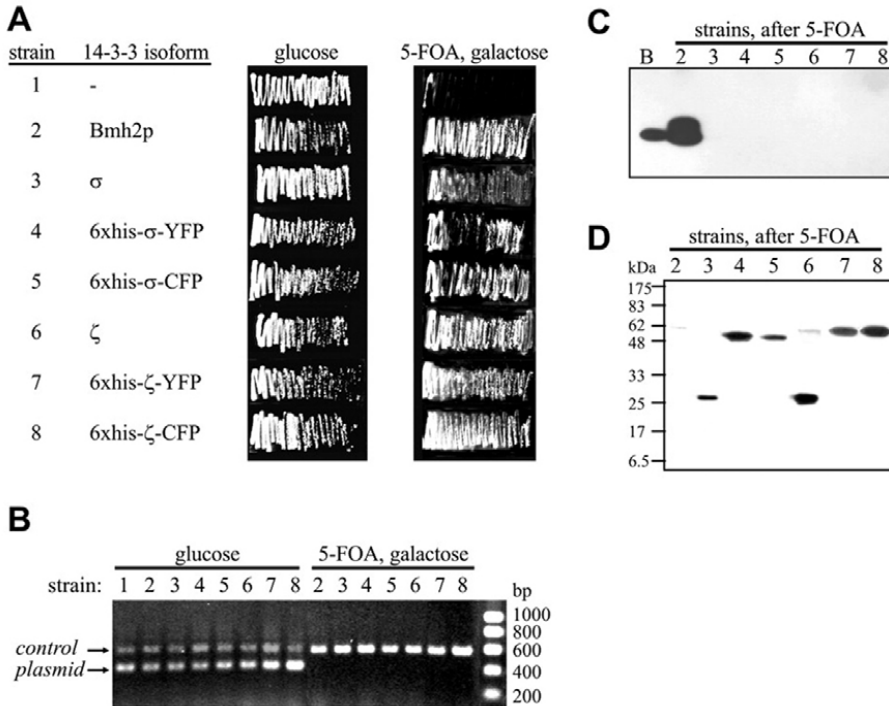


Figure. 1. Recombinant human 14-3-3 proteins complement the lethal disruption of yeast 14-3-3 genes. (A) GG1306-derived yeast strains expressing the indicated 14-3-3 isoforms were transferred from glucose medium (left) to 5-FOA-containing medium (right). Strains can only grow on this medium when they are able to lose plasmid YCplac33[BMH2], illustrating the ability of the foreign 14-3-3 isoform to take over the function of the yeast 14-3-3 proteins. (B) The absence of plasmid Ycplac33[BMH2] after 5-FOA treatment was determined by PCR. This plasmid yields a 400 bp product, besides a 600 bp internal control product. Western blots on 5 μ g (C) or 2.5 μ g (D) total protein from strains after 5-FOA treatment with an antiserum against yeast 14-3-3 proteins (C) or against Pan 14-3-3 proteins (D). Lane b contains 20 ng purified Bmh2p.

14-3-3 σ constructs had slightly lower expression levels compared with 14-3-3 ζ constructs, which might explain the slower growth of strains complemented by 14-3-

3 σ . Expression levels of the recombinant 14-3-3s were estimated to be 1-2% of total protein. These results show that solely the human recombinant 14-3-3 isoforms are responsible for the complementation of the lethal *bmh1 bmh2* disruption in yeast cells. Our data indicate that human recombinant 14-3-3 proteins are functional in yeast and that an N-terminal 6xHis-tag and a C-terminal CFP or YFP fusion do not interfere with at least the essential 14-3-3 function(s).

6xHis-14-3-3 σ -CFP forms dimers and associates with a Raf-1-based peptide

6xHis-tagged CFP and YFP fusions of 14-3-3 σ and 14-3-3 ζ were purified from yeast to a purity of more than 95%. The proteins had a molecular mass of 57 kDa in SDS-PAGE (Fig. 2A), which corresponds to the predicted molecular mass of a monomer. Gel filtration of purified 6xHis-14-3-3 σ -CFP yielded an elution profile with a single peak corresponding to a mass of 116 kDa (Fig. 2B).

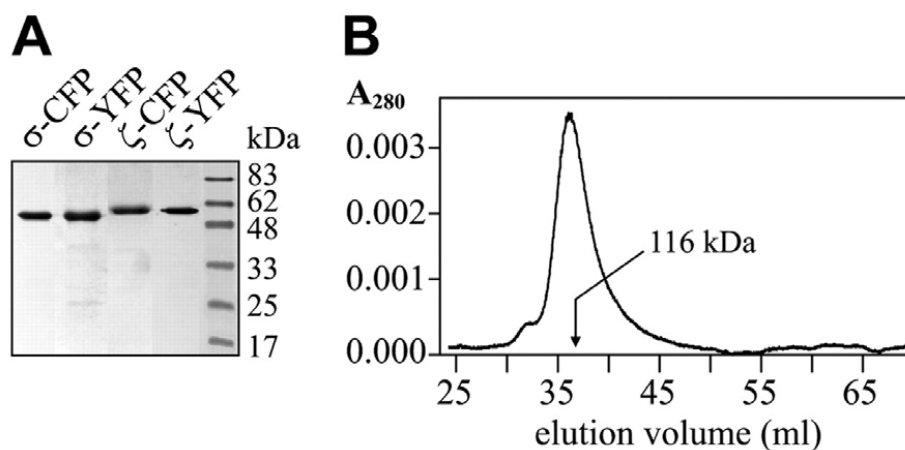


Figure. 2. Recombinant human 14-3-3 isoforms form dimers. (A) Coomassie-stained polyacryl-amide gel of recombinant human 14-3-3 isoforms purified from yeast. (B) Gel filtration analysis of 6xhis-14-3-3 σ -CFP.

This indicates that the protein forms dimers and that dimerisation is not affected by the introduction of an N-terminal 6xHis-tag near the dimerisation domain. For the other 14-3-3 fusion proteins similar results were obtained (data not shown).

Both 14-3-3 σ -YFP and 14-3-3 ζ -YFP bound a Raf-1-based peptide with similar affinity as the yeast 14-3-3 protein Bmh2p in fluorescence correlation spectroscopy measurements (R. Schmauder, unpublished). This suggests that the C-terminal YFP fusion does not interfere with 14-3-3-ligand interactions.

14-3-3 σ and 14-3-3 ζ have a different subcellular distribution

To study the subcellular distribution of 14-3-3 isoforms we transiently transfected various mammalian cell types and determined the localisation of 14-3-3 σ -YFP and 14-3-3 ζ -YFP. Control experiments, in which transfected and untransfected cells were immunostained, showed that transfection had no effect on the distribution of endogenous 14-3-3 σ and 14-3-3 ζ (data not shown). In all cell types investigated, 14-3-3 σ had a mainly diffuse cytoplasmic distribution and only low levels of fluorescence were observed in the nucleus (Fig. 3A, left panel). The intracellular distribution of 14-3-3 ζ -YFP was clearly different. A diffuse cytoplasmic and nuclear localisation was observed, although fluorescence in the nucleus was slightly lower than in the cytoplasm (Fig. 3A, right panel). Nuclear (F_N) and cytoplasmic (F_C) 14-3-3 levels were determined and the ratio F_N/F_C was calculated for each cell type (Fig. 3B). 14-3-3 σ and 14-3-3 ζ had a clearly distinct intracellular distribution in all cell types investigated. The F_N/F_C ratio of 14-3-3 ζ was about twice that of 14-3-3 σ . Immunolocalisation of endogenous 14-3-3 σ and 14-3-3 ζ with specific antibodies in HaCaT and Hela cells revealed similar subcellular distributions as were found with the 14-3-3-YFP constructs (Fig. 3B; Fig. 3C, hatched bars). This indicates that results obtained with YFP-labelled 14-3-3 isoforms have biological significance.

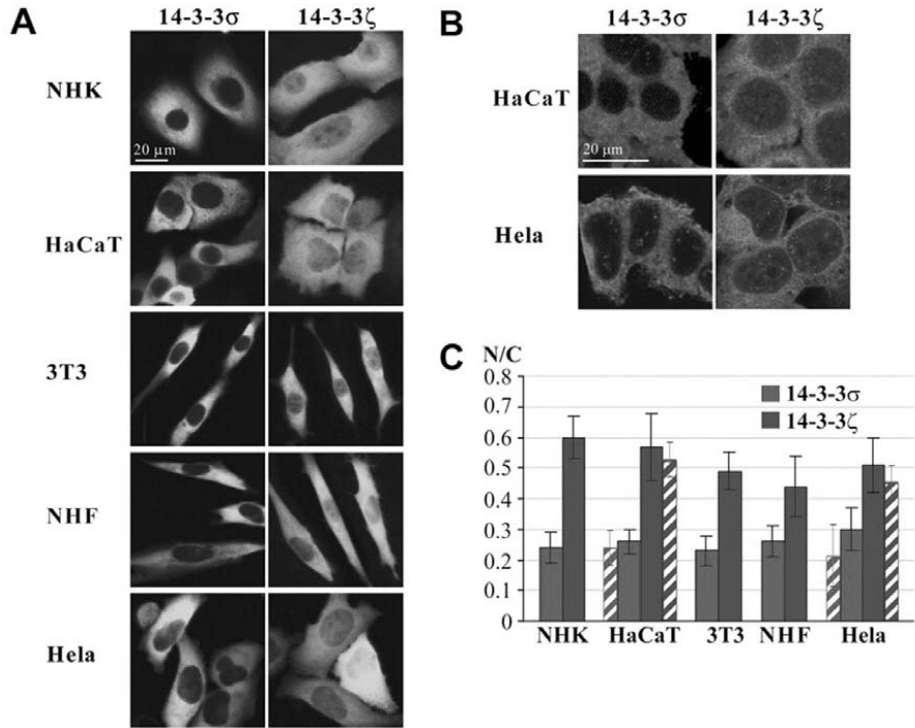


Figure. 3. (A) Subcellular distribution of 14-3-3 σ -YFP and 14-3-3 ζ -YFP in the indicated mammalian cells. (B) HaCaT and HeLa cells immunostained for endogenous 14-3-3 σ and 14-3-3 ζ . (C) Quantitative analysis of the subcellular distribution of 14-3-3 σ (light) and 14-3-3 ζ (dark). Solid bars represent the average nuclear/cytoplasmic (F_N/F_C) ratio obtained with YFP-labelled 14-3-3 isoforms. Hatched bars represent the quantitative analysis of immunolocalisations of endogenous 14-3-3 σ and ζ . Standard deviations are indicated (black lines).

We further investigated the subcellular distribution of 14-3-3 by biochemical fractionation of HaCaT cells into a cytosolic and nuclear extract. Equal amounts of total protein from both extracts were analysed by western blotting with specific antibodies for 14-3-3 σ (Fig. 4A) and 14-3-3 ζ (Fig. 4B). 14-3-3 σ appears to be mainly cytosolic, although a small amount can be seen in the nucleus (not visible in this exposure) when more protein is loaded (data not shown). The majority of 14-3-3 ζ is cytosolic, but a significant amount is present in the nucleus (Fig. 4B). A control experiment with antibodies against known nuclear (p53), cytoplasmic (RasGAP, PKC) and cytoskeletal (actin, α -tubulin) proteins shows that the nuclear extract is not contaminated with cytoplasmic or cytoskeletal proteins and that the cytosolic fraction

is not contaminated with nuclear proteins (Fig. 4C). These data confirm the results from microscopic analysis.

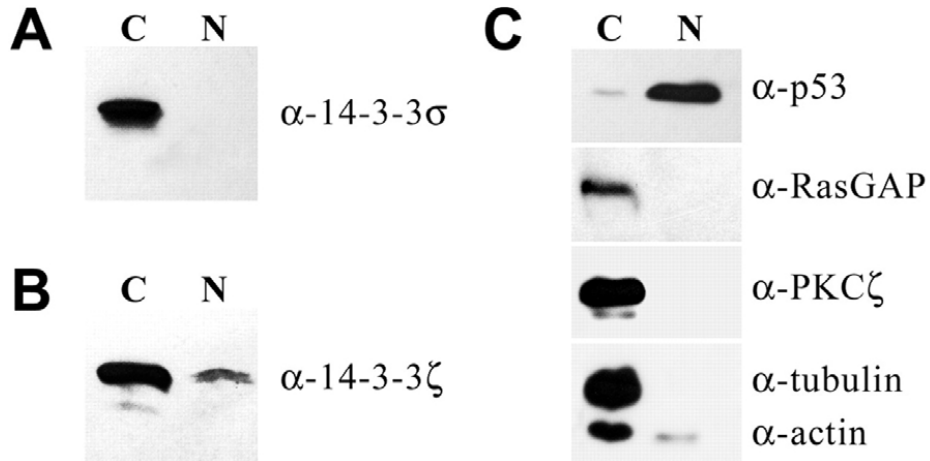


Figure. 4. Subcellular distribution of 14-3-3 after biochemical cell fractionation. HaCaT cells were fractionated into a cytosolic (C) and nuclear extract (N). Equal amounts of total protein from both extracts were analysed by western blotting. (A) Blot stained with specific 14-3-3 σ antibodies or (B) specific 14-3-3 ζ antibodies. (C) Control probed with antibodies against known nuclear (p53), cytoplasmic (RasGAP, PKC) and cytoskeletal (actin, -tubulin) proteins.

Crm1-dependent export of 14-3-3 σ and 14-3-3 ζ

Both 14-3-3 σ and 14-3-3 ζ contain a putative nuclear export sequence (NES). Therefore, we studied the effect of the Crm1-dependent nuclear export inhibitor LMB on the intracellular 14-3-3 distribution. Treatment of HaCaT cells expressing 14-3-3 σ -YFP with LMB led to a clear increase in nuclear 14-3-3 σ concentration (Fig. 5A). For 14-3-3 ζ , a similar increase in nuclear concentration was observed, although the effect appeared to be less strong. The nuclear accumulation of 14-3-3 σ and 14-3-3 ζ had already reached its maximum 2 hours after LMB treatment and no significant changes were observed 4, 5 and 24 hours after the addition of LMB. A fraction of cells appeared relatively unresponsive to LMB treatment. We calculated the F_N/F_C ratio for 50 HaCaT cells expressing either 14-3-3 σ -YFP or 14-3-3 ζ -YFP, with and without LMB treatment (Fig. 5B). Untreated cells expressing 14-3-3 σ -YFP had an average F_N/F_C of 0.31 ± 0.07 . Subsequently, the percentage of cells with an F_N/F_C ratio higher than 0.45 (average F_N/F_C plus two times standard deviation of untreated

cells) was calculated (Fig. 5C). Untreated cells expressing 14-3-3 ζ -YFP had an average F_N/F_C of 0.59 ± 0.09 , and therefore we calculated the percentage of cells with $F_N/F_C > 0.77$ for 14-3-3 ζ (Fig. 5C).

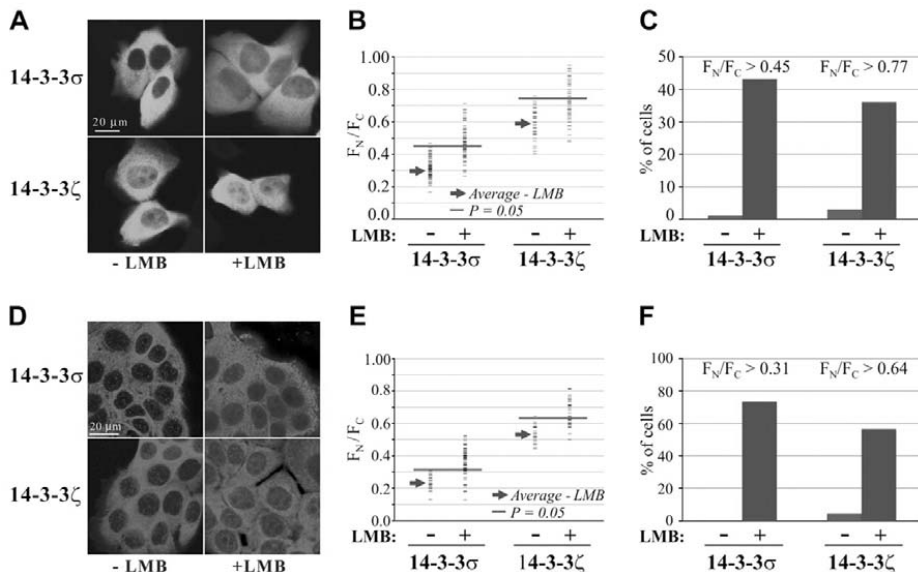


Figure 5. Effect of LMB on intracellular 14-3-3 σ and 14-3-3 ζ distribution. (A) HaCaT cells expressing 14-3-3 σ -YFP or 14-3-3 ζ -YFP were incubated in medium with or without LMB for 5 hours. (B) Plot of the individual F_N/F_C ratios for 14-3-3 σ -YFP and 14-3-3 ζ -YFP of 50 cells, with and without LMB treatment. (C) Percentage of cells with a significantly increased F_N/F_C ratio ($F_N/F_C > 0.45$ for 14-3-3 σ and $F_N/F_C > 0.77$ for 14-3-3 ζ). (D) HaCaT cells incubated in medium with or without LMB for 5 hours were immunostained with specific antibodies for 14-3-3 σ or 14-3-3 ζ . (E) Plot of the individual F_N/F_C ratios for endogenous 14-3-3 σ and 14-3-3 ζ of cells treated with and without LMB. (F) Percentage of cells with a significantly increased F_N/F_C ratio of endogenous 14-3-3 ($F_N/F_C > 0.31$ for 14-3-3 σ and $F_N/F_C > 0.64$ for 14-3-3 ζ).

To study the effect of LMB treatment on the subcellular distribution of endogenous 14-3-3 proteins, we immunostained untreated and LMB-treated HaCaT cells with antibodies specific for 14-3-3 σ or 14-3-3 ζ (Fig. 5D). LMB treatment led to a noticeable increase in nuclear 14-3-3 concentration. As described above, we determined the F_N/F_C ratio for individual cells immunostained for either 14-3-3 σ or 14-3-3 ζ , with and without LMB treatment (Fig. 5E). Subsequently, the percentage of cells with an F_N/F_C ratio higher than 0.31 for 14-3-3 σ and higher than 0.64 for 14-3-

3 ζ was calculated (Fig. 5F). As was found with the YFP-labelled isoforms, LMB treatment appeared to have a stronger effect on the nuclear accumulation of 14-3-3 σ than that of 14-3-3 ζ .

Our data show that LMB treatment leads to a significant increase in nuclear 14-3-3 levels, indicating that 14-3-3 is exported at least in part by a NES- and Crm1-dependent mechanism. The fact that LMB treatment leads to a significant, but not complete accumulation of 14-3-3 in the nucleus, suggests that, besides Crm1-dependent export, other export mechanisms are also involved. A fivefold higher LMB concentration did not result in increased nuclear accumulation (data not shown), which is in line with the findings of others that the LMB concentrations used in this study are sufficient to give an almost complete inhibition of Crm1.

Nuclear import and export rates of 14-3-3 σ and 14-3-3 ζ

Why do 14-3-3 σ and 14-3-3 ζ have a different subcellular distribution? 14-3-3 σ is either imported more slowly or exported more rapidly than 14-3-3 ζ . To investigate this aspect, we studied the nuclear import and export of 14-3-3 σ and 14-3-3 ζ using FRAP in the presence and absence of LMB. A region covering >75% of the nucleus of HaCaT cells expressing 14-3-3 σ -YFP, 14-3-3 ζ -YFP or YFP alone was photobleached for 3 seconds, after which fluorescence recovery in the nucleus was followed over time (Fig. 6A). Photobleaching a square region in the nucleus for 3 seconds did not lead to a discrete square photobleached zone, but resulted in a homogenous decrease in fluorescence throughout the entire nucleus (Fig. 6A). This shows that the 14-3-3 proteins are highly mobile within the nucleoplasm and therefore that the observed relatively slow fluorescence recovery in the nucleus is the result of exchange of photobleached molecules for fluorescent molecules from the cytoplasm. Time-dependent recovery of nuclear fluorescence was quantified and the nuclear import (k_i) and export (k_e) rate constants and half-times ($1/2$) were determined as described in Materials and Methods. Repeated photobleaching of the nucleus of the same cell yielded similar half-times (data not shown), indicating that the photobleaching process did not affect the import-export process.

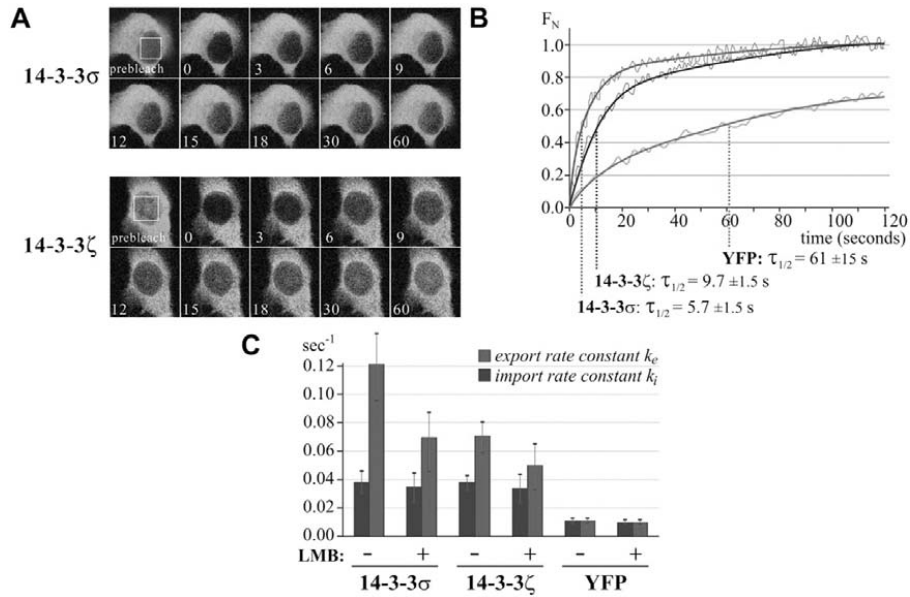


Figure 6. FRAP analysis of nucleocytoplasmic transport of 14-3-3 proteins. (A) A region covering >75% of the nucleus (indicated by the white box) of HaCaT cells expressing 14-3-3 σ -YFP or 14-3-3 ζ -YFP was photobleached, after which images were recorded at 1-second intervals. Fluorescence recovery is shown for two representative cells at the indicated times in seconds. (B) Typical normalised recovery curves with $\tau_{1/2}$ for nuclear export for 14-3-3 σ -YFP, 14-3-3 ζ -YFP and YFP alone. (C) Rate constants for the nuclear import and export of 14-3-3 σ -YFP, 14-3-3 ζ -YFP and YFP alone, in untreated and LMB-treated cells. At least 15 cells were measured and quantified in each experiment.

Typical normalised recovery curves for 14-3-3 σ -YFP, 14-3-3 ζ -YFP and the YFP control are shown in Fig. 6B. A $\tau_{1/2}$ of 5.7 ± 1.5 seconds and 9.7 ± 1.5 seconds was found for the nuclear export of 14-3-3 σ -YFP and 14-3-3 ζ -YFP, respectively. For nuclear import, a $\tau_{1/2}$ of 18 seconds was found for both 14-3-3 σ -YFP and 14-3-3 ζ -YFP. As the k_e of 14-3-3 σ is 1.7 times higher than the k_e of 14-3-3 ζ , while the k_i s are equal, the different subcellular distribution of 14-3-3 σ compared with 14-3-3 ζ appears to be caused by the more efficient export of 14-3-3 σ . Comparing these values to the $\tau_{1/2}$ of 61 ± 15 seconds for the passive diffusion of YFP alone suggests that the 14-3-3 proteins shuttle rapidly in and out of the nucleus through active transport. To ensure that the results from our FRAP experiments reflect nuclear export of 14-3-3, we performed FRAP experiments on cells treated with the nuclear export inhibitor LMB. Fig. 6C shows the k_i s and k_e s for 14-3-3 σ -YFP, 14-3-3 ζ -YFP and

YFP alone in untreated and LMB-treated cells. LMB treatment reduced the k_e for 14-3-3 σ export by 45% and by 30% for 14-3-3 ζ (Fig. 6C). The k_{is} (calculated from the k_e and the increased F_N/F_C) were not significantly reduced after LMB treatment. This suggests that 14-3-3 σ and, to a lesser extent, 14-3-3 ζ are exported at least in part by a Crm1-dependent mechanism.

Discussion

Nucleocytoplasmic transport of proteins plays an important role in the regulation of many cellular processes. Differences in the kinetics of nuclear shuttling can provide a basis for isoform-specific functional specialisation of members of multigene families. This is particularly significant for conserved protein families, like the 14-3-3 proteins. Many organisms contain multiple 14-3-3 isoforms and, thus far, it remains unclear whether and to what extent these isoforms have specialised biological functions and whether these are based on temporal and tissue-specific expression regulation and/or on isoform-specific ligand binding. In addition, ambiguous and conflicting data have been reported on the specific subcellular distribution of isoforms (Roth et al., 1994; Martin et al., 1994; Sehne et al., 2000; Pietromonaco et al., 1996). To gain more insight in this matter, we studied the subcellular distribution and dynamic localisation of 14-3-3 σ and 14-3-3 ζ in living mammalian cells.

We first showed that recombinant human 14-3-3-YFP constructs are functional *in vivo* in yeast, that they form dimers *in vitro* and are able to bind a Raf-1-based peptide. This suggests that the fusion of YFP to the C-terminus and a 6xHis-tag to the N-terminus of 14-3-3 does not interfere with the major 14-3-3 functions.

The subcellular distribution of 14-3-3 σ and 14-3-3 ζ was studied in various mammalian cell types by immunostaining endogenous 14-3-3 proteins with specific antibodies and by analysis of cells transiently expressing YFP fusions of these proteins. Biochemical fractionation of HaCaT cells provided additional insight in the isoform-specific subcellular distribution of the 14-3-3 proteins. 14-3-3 σ exhibited a mainly diffuse cytoplasmic distribution and was present at low levels in the nucleus.

But nuclear concentrations of 14-3-3 ζ -YFP were noticeably higher in comparison with 14-3-3 σ . F_N/F_C ratios of 0.26 and 0.52 were found for 14-3-3 σ and 14-3-3 ζ , respectively. No significant differences in subcellular distribution were found between the various cell types, indicating that the mechanism responsible for the more efficient nuclear exclusion of 14-3-3 σ is unlikely to be cell-type specific, as it is even present in cell types that normally do not express 14-3-3 σ (e.g. NIH-3T3 and NHF).

We performed FRAP experiments on living cells to study the mechanism underlying the different subcellular distributions of 14-3-3 σ and 14-3-3 ζ . Both 14-3-3 σ and 14-3-3 ζ rapidly shuttle in and out of the nucleus through active transport. 14-3-3 σ had a 1.7x higher nuclear export rate constant than 14-3-3 ζ , whereas import rate constants were equal. Therefore, the difference in subcellular distribution between 14-3-3 σ and 14-3-3 ζ appeared to be caused by the more efficient export of 14-3-3 σ . Treatment of cells with LMB led to an increase in nuclear 14-3-3 levels and to a significant decrease of the export rate constants of both 14-3-3 σ and 14-3-3 ζ , suggesting that these proteins are exported at least in part by a Crm1-dependent mechanism.

One possible explanation for the different subcellular distributions and export rates of 14-3-3 σ and 14-3-3 ζ might rely on the finding that nuclear export of 14-3-3 could be governed by binding partners. In this model, 14-3-3 itself plays no active role in nuclear export and is exported out of the nucleus via a 'piggy-back' mechanism in which the binding partner provides the NES (Brunet et al., 2002). Isoform-specific ligand interactions would in this case cause the different subcellular distributions of 14-3-3 σ and 14-3-3 ζ . 14-3-3 might preferentially bind ligands that are rapidly exported from the nucleus. This model, however, does not explain why 14-3-3 is required for the nuclear export of some binding partners (Wang and Yang, 2001; Brunet et al., 2002).

An alternative model is that sequences in 14-3-3 itself govern nuclear export. It has been reported that a putative NES is present in the C-terminus of 14-3-3, in a region that also appeared to be involved in ligand binding (Lopez et al., 1999; Rittinger et al., 1999). Although this sequence was able to function as NES when fused to GFP, a recent study suggests that this region is only involved in ligand binding (Brunet et al., 2002). However, one could envisage that the NES of 14-3-3 is

(partly) folded into the ligand-binding groove in the absence of a binding partner, leading to nuclear retention of unbound 14-3-3. In support of this, the C-terminus appears to function in the inhibition of improper 14-3-3-ligand interactions by occupying the ligand binding groove (Truong et al., 2002). On ligand binding, the NES might be displaced from the groove and become exposed, leading to nuclear export through Crm1. In such a model, unbound 14-3-3 would home to the nucleus, where after ligand binding its NES is exposed, leading to rapid translocation to the cytoplasm. This model would explain why 14-3-3 is required for the nuclear export of certain ligands (Brunet et al., 2002). As 14-3-3 σ and 14-3-3 ζ both contain an identical NES consensus motif (216-DLIMQLLRDNLTLW) in their C-termini, it is unlikely that this region is responsible for the difference in subcellular distribution of these isoforms. However, the extreme C-terminal region directly flanking the putative NES, which might be involved in the structural changes mentioned above, is highly divergent and could account for the observed isoform specificity. Clearly, more experiments, which are beyond the focus of this analysis, are needed to elucidate the molecular mechanisms that underlie the isoform-specific export rates that we have measured.

In conclusion, we have shown differences in the subcellular distribution and nuclear export kinetics of 14-3-3 proteins, which might be the basis for isoform-specific biological functions. Our data indicate that one should not think of the 14-3-3 proteins as static molecules that passively sequester ligands in the cytoplasm. On the contrary, 14-3-3 proteins rapidly shuttle in and out of the nucleus, making fast responses to signals possible and allowing them to play an active role in changing the localisation of binding partners. The rapid nuclear export of 14-3-3 σ in particular is interesting, as it might reflect a mechanism by which cells can efficiently control the nuclear exclusion of proteins involved in cell cycle progression. The high export rates of 14-3-3 σ are in line with a transient function of this protein in the nucleus and with the proposed role of 14-3-3 σ in causing rapid DNA damage-induced cell cycle arrest through its p53-mediated upregulation and cytoplasmic sequestering of Cdc2 (Laronga et al., 2000; Chan et al., 1999; Hermeking et al., 1997). The fact that YFP fusion constructs are functional and have the same subcellular distribution as endogenous 14-3-3 isoforms creates new and interesting possibilities for future

functional studies on 14-3-3 proteins in living cells. Our findings might reflect a mechanism that can be the basis for isoform-specific specialisations within multigene families in general.

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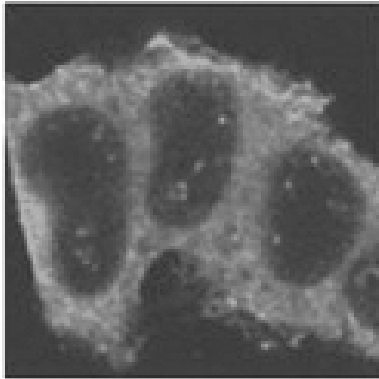
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Chapter 5



Radiation response and cell cycle regulation of p53 rescued malignant keratinocytes

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Abstract

Mutations in the tumor suppressor gene p53 were found in more than 90% of all human squamous cell carcinomas (SCC). To study the function of p53 in a keratinocyte background, a tetracycline-controlled p53 transgene was introduced into a human SCC cell line (SCC15), lacking endogenous p53. Conditional expression of wild-type p53 protein upon withdrawal of tetracycline was accompanied with increased expression of p21^{WAF1/Cip1} resulting in reduced cell proliferation. Flow-cytometric analysis revealed that these cells were transiently arrested in the G1/S phase of the cell cycle. However, when SCC15 cells expressing p53 were exposed to ionizing radiation (IR), a clear shift from a G1/S to a G2/M cell cycle arrest was observed. This effect was greatly depending on the presence of wild-type p53, as it was not observed to the same extent in SCC15 cells lacking p53. Unexpectedly, the p53 and IR dependent G2/M cell cycle arrest in the keratinocyte background was not depending on increased expression or stabilization of 14-3-3 σ , a p53 regulated effector of G2/M progression in colorectal cancer cells. In keratinocytes, 14-3-3 σ (stratifin) is involved in terminal differentiation and its cell cycle function in this cell type might diverge from the one it fulfils in other cellular backgrounds.

Keywords: TP53, 14-3-3 σ , stratifin, p21^{WAF1/Cip1}, cell-cycle checkpoint, DNA damage, keratinocyte, ionizing radiation

Introduction

Mutations in the p53 tumor suppressor gene are found in more than 50% of human epithelial cancers (Hainaut et al., 1997), resulting in either lack of expression, expression of a "loss of function" protein, or increased expression of a mutant p53 protein that can act as a "gain of function" oncoprotein (Dittmer et al., 1993). P53 appears to be especially important in protecting keratinocytes from malignant transformation, since p53 mutations have been found in more than 90% of squamous cell carcinomas (SCC), which are derived from the keratinocyte lineage (Leffell, 2000). The cell cycle regulatory function of p53 is believed to maintain genomic stability following cellular stress and loss of this function promotes malignant transformation (Lane, 1992). The p53 protein is a sequence-specific transcriptional activator of several genes, including the p21^{WAF1/Cip1} and the 14-3-3 σ genes, which are involved in the negative regulation of cell cycle progression in response to radiation or other types of cellular stress (Hermeking et al., 1997; Jongmans & Hall, 1999). The p21^{WAF1/Cip1} protein plays an essential role in p53-mediated G1/S cell cycle arrest by acting as an inhibitor of G1 cyclin-dependent kinases (Harper et al., 1993). The findings that ectopic expression of p21^{WAF1/Cip1} results in G1/S arrest in several cell types (Harper et al., 1995; Medema et al., 1995; Qin & Ng, 2001) and that p21^{WAF1/Cip1} deficient cells fail to arrest in G1/S after DNA damage (Maeda et al., 2002) argue that the G1/S arrest observed in cells expressing wild-type p53 is a direct result of induction of p21^{WAF1/Cip1}. In colorectal cancer cells, the 14-3-3 σ protein contributes to p53-mediated G2/M cell cycle arrest by inhibiting the activation of the G2-cyclin-dependent kinase cdc2 (Chan et al., 1999; Hermeking et al., 1997). HCT116 cells overexpressing exogenous 14-3-3 σ arrest in G2. In response to ionizing radiation (IR) 14-3-3 σ is induced in several colorectal cancer cell lines in a p53-dependent fashion, and ectopic expression of p53 in HCT116 cells results in an 18 fold induction of 14-3-3 σ (Hermeking et al., 1997). 14-3-3 σ deficient HCT116 cells fail to sequester the cdc2-cyclinB1 complex in the cytoplasm after DNA damage. After treatment with adriamycin these cells initially arrest in G2 phase, but are unable to maintain cell-cycle arrest and die as they enter mitosis by a process

called mitotic catastrophe (Chan et al., 1999). In contrast, 14-3-3 σ was shown to play a positive role in IGF-I receptor induced cell proliferation in the human breast cancer cell line MCF7 (Zhang et al., 2004). Expression of 14-3-3 σ is highly cell-type specific, in fact 14-3-3 σ was originally described as the epithelial cell specific protein stratifin (Leffers et al., 1993). Accordingly, 14-3-3 σ is not expressed in either exponentially growing or γ -irradiated human diploid fibroblasts or human endothelial cells (Hermeking et al., 1997). In human epidermal keratinocytes 14-3-3 σ is expressed at low levels in basal cells, at high levels at the moment of commitment to terminal differentiation and again at low levels at later stages of differentiation (Dellambra et al., 2000). Keratinocytes arise from stem cells in the basal layer and move upwards towards the skin's periphery as they differentiate and give rise to the various layers of the epidermis (Alonso & Fuchs, 2003; Roop, 1995). Down-modulation of 14-3-3 σ forces primary human keratinocytes into the stem cell compartment, allowing them to escape from replicative senescence and to become immortal (Dellambra et al., 2000).

In order to determine the role of p53 and the involvement of 14-3-3 σ and p21^{WAF1/Cip1} in the regulation of cell cycle progression in keratinocytes, we used the human squamous cell carcinoma cell line SCC15 (Rheinwald & Beckett, 1980), which does not express endogenous p53 protein (Burns, 1993; Min et al., 1994). Conditional expression of wild-type p53 protein in SCC15 cells resulted in transactivation of the p21^{WAF1/Cip1} gene and a transient arrest of the cells in the G1/S phase of the cell cycle. Conversely, both wild-type p53 expression and exposure to ionizing radiation (IR) were required to efficiently arrest cells in G2/M. In contrast to the findings in colorectal cancer cell lines, this G2/M cell cycle arrest was not depending on up-regulation or stabilization of 14-3-3 σ .

Materials and methods

Cell culture

The SCC15 cell line (generously supplied by Dr. Jim Rheinwald, Cambridge, MA) was derived from a squamous carcinoma of the tongue and grown in standard

medium (calcium concentration 1.4 mM) containing DMEM and Ham's F12 (Gibco/BRL) in a 3:1 ratio, supplemented with 5% bovine calf serum (Hyclone) and 0.4 µg/ml hydrocortisone (Sigma) (Rheinwald & Beckett, 1980). For tetracycline-regulated gene induction experiments Tet-system-approved fetal bovine serum (Clontech) was used (Tet-free medium). SCC15 cell lines and derivatives were cultured in a humidified incubator at 8% CO₂ and 37°C unless indicated differently. Cells were irradiated with X-rays using an Andrex SMART-225 device. MG132 was purchased from Calbiochem® and used as indicated. The retroviral packaging cell line Phoenix (a gift from Dr. G. Nolan, Stanford) was maintained at 5% CO₂ and 37°C in DMEM supplemented with 10% bovine calf serum (Nolan & Shatzman, 1998).

Vectors

The pTEV retroviral vector used in this study was based on the self-inactivating SFGtcLucITE1 retroviral vector (Lindemann et al., 1997). 147 bp enhancer sequences in the 3' LTR of this retroviral vector have been deleted to decrease basal LTR-mediated gene transcription when the retroviral vector is integrated in the genome of the target cells. The pTEV1 retroviral vector was created by replacing the NcoI-SacII (containing the luciferase and tTA genes) by a fragment harbouring a T₇ promoter sequence followed by multiple unique cloning sites (NcoI-MluI-BamHI-NotI-ClaI-SacII). To obtain pTEV2, the NcoI site from pTEV1 was deleted. Genes inserted into the unique multiple cloning sites of the pTEV retroviral vectors are transcriptionally controlled by the CMV promoter that contains a binding site for the tetracycline-responsive transactivator (tTA). To create pTEVp53, the p53 gene derived from pC53-SN3 (Baker et al., 1990) was cloned as a BamHI fragment into pTEV2. The control pTEVLuc retroviral vector was obtained by introducing the NcoI-BamHI fragment from the SFGtcLucITE1 into the NcoI-BamHI sites of pTEV1. The tetracycline-responsive transactivator (tTA) was expressed from pPurTet-Off. This plasmid was derived from pRetro-off (Clontech) by replacing the Neo gene by the puromycin-N-acetyl-transferase (pac) selection marker.

The Luciferase reporter plasmid p21Luc was constructed by insertion of the human p21^{WAF1/Cip1} promoter derived from WWP-Luc (el-Deiry et al., 1993) in front of

the luciferase gene of the pGL3-basic plasmid (Promega). The pRSV-p53^{V143A} and pRSV-p53wt expression vectors were constructed by inserting the BamHI fragment from respectively pC53-SCX3 and pC53-SN3 (Baker et al., 1990) into the unique BamHI site of the expression vector pRSV-H2O (gift from Dr. R. Offringa, Leiden).

Transient transfections

Cells grown to 80-90% confluence on 5 cm dishes were transiently transfected by using the liposome transfection reagent DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate) (Roche). 5 µg of luciferase reporter plasmid or a mixture of 5 µg of luciferase reporter plasmid and 0.5 µg of RSV-driven expression vector were added to 1 ml of calcium-free DMEM. 25 µg DOTAP was added gently and the mixture was incubated for 20 minutes at room temperature. Cells washed twice with phosphate-buffered saline (PBS) were supplied with the DNA-DOTAP mixture and incubated for 2 hours at 37°C with occasional rocking of the culture dishes. Subsequently, the cells were washed once with PBS and fresh standard medium was added. 24 hours after transfection the cells were rinsed with cold PBS and collected by scraping in 500 µl PBS. The cells were resuspended and sonicated in 150 µl cell lysis buffer (Promega). Following centrifugation, the luciferase activity of the cleared supernatant was determined in the presence of luciferin (Promega) and expressed as relative light units/µg protein.

Retrovirus-mediated Infections

Retroviral vectors were transiently transfected into the amphotropic Phoenix packaging cell line as described by Dr. G. Nolan (<http://www.stanford.edu/group/nolan>). Transiently produced retroviruses were collected after 48 hours, filtered (0.45 µm; Sarstedt) and added to SCC15 cells in the presence of 5 µg/ml polybrene. SCC15 cell lines in which the expression of the gene of interest can be regulated by the tetracycline-responsive transactivator (tTA) were established in two steps. First, pPurTet-Off containing the tetracycline-responsive transactivator (tTA) was introduced into SCC15 cells by retroviral transduction. After 24 hours incubation at 32°C medium was replaced by fresh standard medium and the temperature increased to 37°C. After another 24 hours resistant clones were selected

using 1 $\mu\text{g/ml}$ puromycin. After 2 weeks the puromycin-resistant SCC15 clones were pooled and grown for 24 hours in the presence of tetracycline (2 $\mu\text{g/ml}$). Infection with pTEV retroviruses was then performed for 24 hours at 32°C, as described above. Individual clones were isolated by dilution cloning and were grown in standard medium containing 1 $\mu\text{g/ml}$ puromycin and 2 $\mu\text{g/ml}$ tetracycline at 37°C and 8 % CO₂.

Cell-cycle analysis

Non-confluent growing cells were trypsinized, washed in PBS, resuspended in Hanks' balanced salt solution buffer (Gibco) and stored at 4°C in 55% ethanol. Before analysis with a FACScalibur flow-cytometer (Becton Dickinson) cells were pelleted, resuspended in 1 ml of PBS solution and treated with RNase A (100 $\mu\text{g/ml}$) for 40 min at 37°C and with propidium iodide (30 $\mu\text{g/ml}$) for 15 min at room temperature. The percentage of cells in various cell cycle phases was determined by DNA histogram analysis of at least 10.000 sorted cells using WinMDI 2.8 software. Results presented in figures 2 and 4 are based on two independent experiments with similar results.

Protein isolation and immunoblotting

Cells grown on 5 cm dishes were washed in PBS and lysed directly on the dishes in cold sample buffer (60 mM TRIS pH 6.8, 10 % glycerol, 2 % SDS, 2 % β -mercaptoethanol, 0.003 % bromophenol blue) and subsequently sheared by passing several times through a 29 gauge needle. Sample solutions were boiled for 5 minutes and loaded on a SDS polyacrylamide gel. After staining the gel with Coomassie brilliant blue and scanning, equal loading was checked by using the Quantity One-4.2.1 (Biorad) program. Adjusted volumes of sample were separated alongside Rainbow marker proteins (Amersham) or Cruz Marker™ (Santa Cruz Biotechnology) using a mini-gel system and transferred to a nitrocellulose membrane using a semi-dry blotting procedure or to a PVDF membrane (Hybond-P Amersham) using the Mini Protean 3 system (Biorad). Blots were incubated with antibodies against p21(F-5), 14-3-3 σ (N-14) or p53(DO-1) (all from Santa Cruz Biotechnology) and detected by the enhanced chemiluminescence detection system (ECL, Amersham) using donkey

anti-Goat IgG-HRP for 14-3-3 σ or donkey anti-Mouse IgG-HRP for p21 and p53 (Santa Cruz Biotechnology).

RNA analysis

Total RNA was isolated from cells grown to 90% confluence by direct lysis on the culture dishes using TRIzol reagent (Invitrogen®) according to the manufacturer's instructions. Equal amounts (20 μ g) of total RNA were separated on a 1% denaturing agarose gel and transferred to a Genescreen membrane (Biotech Systems Dupont, Boston, USA). The Northern blot was hybridized with ³²P-dCTP labeled probes as described (Belt et al., 1989). The p53-specific cDNA probe was derived from the pC53-SN3 vector excised with BamHI (Baker et al., 1990). The p21^{WAF1/Cip1} specific cDNA probe was generously provided by Dr. M. Ozturk (INSERM). The 14-3-3 σ specific probe was made by PCR from a keratinocyte cDNA library by using primers: 5'-CCGATGGATCCATGGAGAGAGCCAGTCTGATC and 5'-CTATGGATCCTCAGCTCTGGGGCTCCTG and was checked by sequencing. Expression levels were quantified using the Quantity One-4.2.1 (Biorad) program and expression of p53, 14-3-3 σ and p21^{WAF1/Cip1} was normalized for the level of GAPDH in the same samples.

RT-PCR

RNA samples were treated with 20 Units of RNase-free DNaseI (Roche) for 30 minutes at room temperature and phenol:chloroform extracted. Total RNA was reverse-transcribed with Super-RT (SphaeroQ) using random hexamer primers (Amersham Pharmacia Biotech). PCR was performed for 30 (Pac, GAPDH, 14-3-3 σ , p53, fig.1) or 35 (p21^{WAF1/Cip1} and p53, fig.6) cycles, with 20 pmol of gene-specific primers, 1.5mM MgCl₂ and 1.25 Units of Amplitaq Gold™ (Roche).

The following unique primer sets were used: pTEV encoded p53 mRNA: 5'-TAATACGACTCACTATAGG-3' and 5'-CTTCCACTCGGATAAGATGC-3'; puromycin-N-acetyl-transferase: 5'-TTCGAATTCAGCTTACCATGACCGAGTA-3' and 5'-AAAGCCGGCTCAATTGTTTTACGCGGCCCG-3'; GAPDH: 5'-CGGAGTCAACGGATTTGGTCGTAT and 5'-AGCCTTCTCCATGGTGGTGAAGAC;

p21^{WAF1/Cip1}: 5'-GTGGACCTGTCACTGTCTTGAC and 5'-CTTCCTCTTGGAGAAGATCAGC (Wedemeyer et al., 2000); 14-3-3 σ 5'-GTCCGGCATTGGTCCCAG and 5'-GTCCCCGGCCTCCTTGAT. PCR products were visualized on a 1.5% agarose gel.

Determination of p53 sequences in various transgenic SCC15 clones

The transgenic clones SCC15TEVp53-11, SCC15TEVp53-19 and SCC15TEVp53-46 were induced in Tet-free medium and RNA was isolated using the TRIzol method. RT-reactions were performed as described above. The p53 coding sequences were amplified as two overlapping fragments. After a 1min hot start at 94°C, PCR was carried out for 35 cycles (20 sec, 94°C; 30 sec, 50°C; 1 min, 72°C) and followed by final elongation at 72°C for 5 minutes. Primers used for the N-terminal fragment were: 5'-TAATACGACTCACTATAGG and 5'-CTTCCACTCGGATAAGATGC and for the C-terminal fragment: 5'-CTTGTTCACTGGAGCCCCG and 5'-GTCTGTGACTTGACGTACTCC. All PCR products were sequenced in two directions and compared to the p53 wild-type sequence (NCBI file: X02469).

Results

Isolation of tetracycline-regulated p53 transgenic SCC15 cells

To investigate the effect of wild-type (wt) p53 protein expression on the behaviour of human keratinocytes, we used the cell line SCC15, which does not express endogenous p53 protein (Burns, 1993; Min et al., 1994). We first examined the effect of transient co-transfection of p53 on the transactivation of the p21^{WAF1/Cip1} promoter in SCC15 cells. Expression of wild-type p53 transactivated the reporter construct p21Luc in SCC15 cells. In contrast, no transactivation of this reporter construct was seen by co-transfecting mutant p53^{V143A} (data not shown).

Having established that ectopically expressed wt p53 is active in SCC15 cells, we generated p53 transgenic SCC15 clones in which p53 gene expression is regulated by the tetracycline-responsive transactivator (tTA) (see Materials & Methods). Upon

withdrawal of tetracycline for 3 days, seven out of forty-four individual SCC15 clones (Fig. 1A) were found to express the p53 gene from the pTEVp53. RT-PCR analysis was performed with a unique primerset that discriminates between the pTEV-encoded and endogenous p53 mRNA. Induced levels of p53 mRNA were detected in most of the SCC15 cells already 1 day after withdrawal of tetracycline (data not shown). However, maximal levels of expression were reached after 3 days (Fig. 1A). Marked differences between the levels of basal and induced p53 mRNA expression were observed in six of the seven isolated SCC15 clones, with the SCC15TEVp53-19 and SCC15TEVp53-46 clones showing the lowest levels of both basal and induced p53 mRNA. Only clone SCC15TEVp53-44 showed similar levels of p53 mRNA in the induced and uninduced states. Identical conclusions could be drawn from Northern blot analysis (data not shown). All clones, except SCC15TEVp53-44, expressed p53 protein upon withdrawal of tetracycline for 3 days (Fig. 1B). Sequence analysis of various transgenes established that clones SCC15TEVp53-19 and SCC15TEVp53-46 expressed wild-type p53, whereas clone SCC15TEVp53-11 contained a glutamic acid to lysine substitution at position 286 of the p53 protein. The wild-type clones SCC15TEVp53-19 and SCC15TEVp53-46 showed a clear transactivation of the reporter plasmid p21Luc (Fig. 1C) whereas no transactivation was observed in SCC15TEVp53-11 and in SCC15TEVp53-44. The lack of transactivation of the p21Luc reporter plasmid, combined with the higher basal p53 gene expression levels, strongly suggests that the p53 transgene has also become mutated in all other clones, similar to the situation in SCC15TEVp53-11.

As a control, puromycine resistant SCC15 cells were also infected with retroviruses containing a tTA-regulated luciferase gene (pTEVLuc). In three clones displaying low levels of luciferase activities in the presence of tetracycline, increased luciferase activities were measured upon withdrawal of tetracycline during 3 days. One of these three clones, SCC15TEVLuc-1 that was further analyzed, showed no expression of p53 upon withdrawal of tetracycline as expected (Fig. 1A).

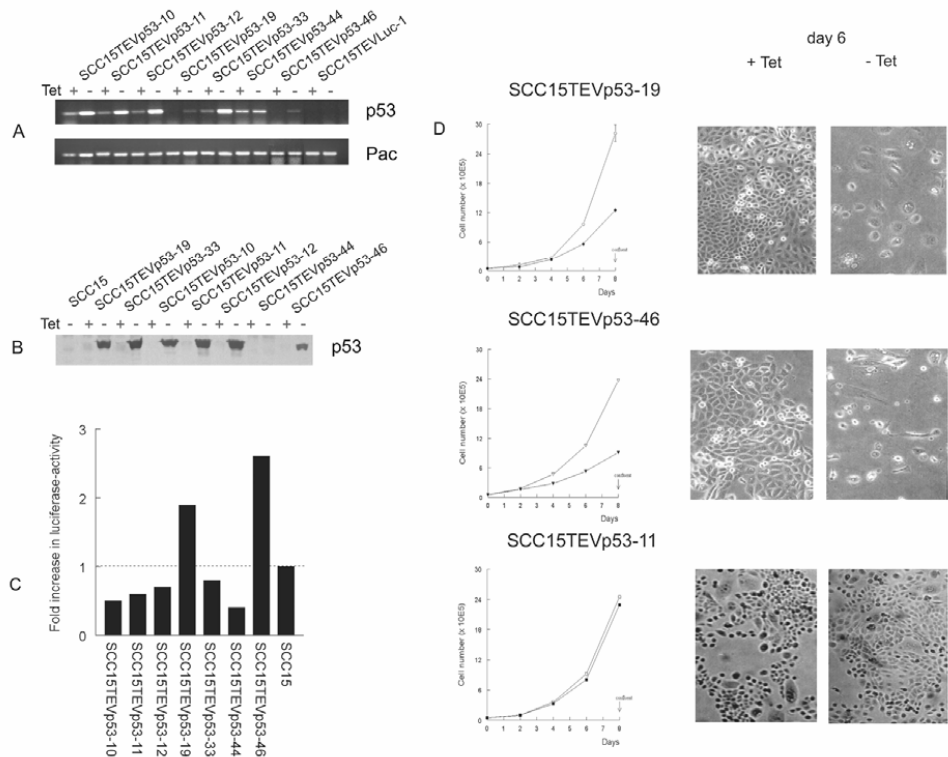


Figure 1. Isolation of SCC15 clones expressing functional Tet-regulated p53. (A) RT-PCR analysis of p53 or Pac (internal control) mRNA, isolated from various SCC15 clones grown for 3 days in medium with or without tetracycline. (B) Immunoblot analysis of induced (-Tet) and uninduced (+Tet) p53 protein levels in SCC15 clones. (C) Transactivation of the p53-responsive p21^{WAF1/Cip1} promoter in SCC15 clones expressing Tet-regulated p53. SCC15 cells grown for 2 days in medium with or without tetracycline were transiently transfected with the p21^{WAF1/Cip1} luciferase reporter construct and cell extracts were prepared 24 hours hereafter. Results are represented as the ratio in luciferase activity between -Tet and +Tet cultures. (D) Growth curves of SCC15TEVp53-19, SCC15TEVp53-46 and SCC15TEVp53-11 cells grown in medium with (open symbols) or without tetracycline (closed symbols). Microscope images of day 6 cultures (+/- Tet) are shown.

Suppression of cell growth following p53 expression in transgenic SCC15 cells

Examination of the growth curves of p53 induced (-Tet) and uninduced (+ Tet) SCC15TEVp53-19, SCC15TEVp53-46 and SCC15TEVp53-11 cells revealed a

significant difference in cell growth between the two states in the SCC15TEVp53-19 and SCC15TEVp53-46 cells (Fig. 1D). The uninduced SCC15TEVp53-19 and SCC15TEVp53-46 cells continued to grow with a doubling time of approximately 48 h, whereas the p53 expressing cells showed a substantial reduction in cell growth. In contrast, no difference in cell growth was found between the two states in the mutant SCC15TEVp53-11 cells (Fig. 1D), indicating that reduced cell growth is due to the expression of active wild-type p53 protein. SCC15TEVp53-19 and SCC15TEVp53-46 cells showed a significantly lower confluence after p53 induction (Fig. 1D, - Tet, right panel) as compared to the uninduced state (Fig. 1D, + Tet, left panel). In contrast, for the mutant SCC15TEVp53-11 clone, the confluence of induced (Fig. 1D, - Tet, right panel) and uninduced (Fig. 1D, + Tet, left panel) cells was very similar. DNA histogram analysis showed that S-phase percentages of SCC15TEVp53-19 cells were halved from 16% to 8% within 2 days following p53 induction (Fig. 2). During the same time the percentage of cells in G1 was increased from 60% to 68% and no changes in G2/M were noticed in SCC15TEVp53-19 cells following p53 induction. In contrast, the relative percentages of G1, S and G2 phases in SCC15TEVLuc-1 cells were not changed significantly after 2 days following tetracycline withdrawal (Fig. 2).

The p53 protein controls the expression of the p21^{WAF1/Cip1} gene, which is involved in cell cycle regulation. Therefore, we analyzed p21^{WAF1/Cip1} RNA expression in SCC15TEVp53-19 and SCC15TEVLuc-1 cell lines (Fig. 3). Uninduced SCC15TEVp53-19 cells showed relatively low levels of p21^{WAF1/Cip1} mRNA, as was also seen in the SCC15TEVLuc-1 cells. However, a strong increase in p21^{WAF1/Cip1} mRNA levels was observed in SCC15TEVp53-19 cells following p53 induction. These p53 induced changes in p21^{WAF1/Cip1} gene transactivation were reflected at the cellular level by a decrease in S-phase, an increase in G1 cells and reduced proliferation (Figs. 1D and 2).

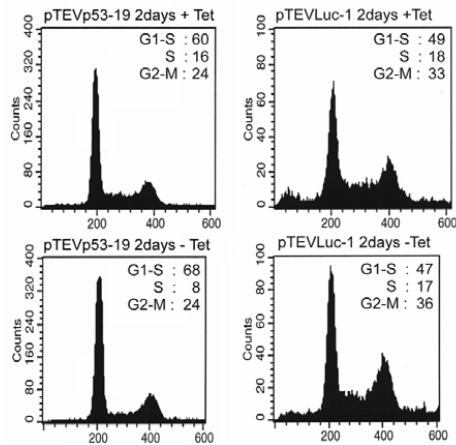


Figure 2. DNA histogram analysis of SCC15TEVp53-19 cells and SCC15TEVLuc-1 cells grown for 2 days in medium with (+Tet) or without (-Tet) tetracycline. The percentages of cells at G1-S, S and G2-M are indicated.

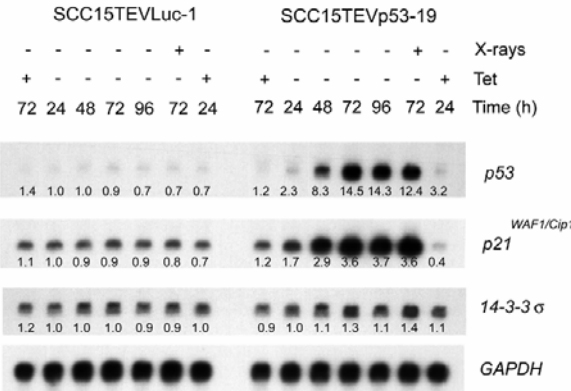


Figure 3. Analysis of p53, p21^{WAF1/Cip1}, 14-3-3σ and GAPDH mRNA levels following a 2 day withdrawal of tetracycline in either SCC15TEVp53-19 or SCC15TEVLuc-1 (control) cells. When indicated cells were additionally exposed to 6 Gy of ionizing radiation at day 1. The indicated relative expression levels were normalized for GAPDH (internal control).

Ionizing radiation (IR) induces p53-mediated G2/M cell cycle arrest in transgenic SCC15 cells

SCC15TEVp53-19 and SCC15TEVp53-11 cells were grown in the presence or absence of tetracycline and subsequently subjected to ionizing radiation (IR) or mock-irradiated. In unirradiated SCC15TEVp53-19 cells, p53 induction resulted in an increase of G1 and a decrease in S-phase cells (Fig. 4, panel A) in accordance with the results presented in figure 2. Irradiation of uninduced SCC15TEVp53-19 cells (+Tet) resulted in a reduction of G1 cells and a moderate increase (16 to 23%) of G2 cells after a 6 Gy dose (Fig. 4, compare +Tet samples in panels A and B). Conversely, when p53 expressing SCC15TEVp53-19 cells (-Tet) were treated with ionizing radiation a more prominent arrest in G2/M was observed. After a dose of 6 Gy the percentage of G2 cells doubled, reaching 34% of the total cell population. (Fig. 4, compare -Tet samples in panels A and B).

Expression of mutant p53 in SCC15TEVp53-11 did not significantly affect the percentage of G2 cells after the same dose of ionizing radiation (Fig. 4, panel D, compare +Tet and -Tet), indicating that IR induced G2/M arrest is greatly depending on the expression of active wild-type p53.

This view is further accentuated by the results of Fig. 4, panel C: after a dose of 10 Gy induction of wild-type p53 increased the fraction of G2 cells from 38% to 56%. Interestingly, at this high dose a pre-G1 peak appeared in SCC15TEVp53-19 cells, which is an indication of apoptosis. This pre-G1 peak was less pronounced in p53 expressing cells (-Tet) than in uninduced cells (+Tet), suggesting that in the presence of wild-type p53 G2/M cell cycle arrest might be favored upon induction of apoptosis.

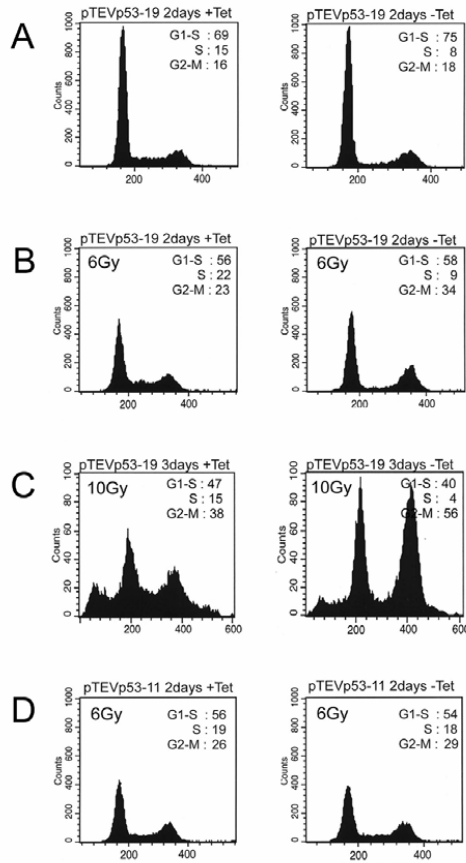


Figure 4. DNA histogram analysis of SCC15TEVp53-19 cells (A,B,C) and SCC15TEVp53-11 cells (D) grown for 2 (A,B,D) or 3 (C) days in +Tet or –Tet medium. When indicated, cultures were exposed to 6 (B,D) or 10 (C) Gy of ionizing radiation at day 1.

14-3-3 σ is not induced or stabilized by p53 in transgenic SCC15 cells

The p53 protein is known to transcriptionally control the expression of the 14-3-3 σ gene. Colorectal cancer cells expressing exogenous p53 showed a significant induction (18 fold) of 14-3-3 σ as compared to the same cells lacking p53, and overexpression of the 14-3-3 σ gene in colorectal cancer cells resulted in an

accumulation of cells in the G2 phase of the cell cycle (Hermeking et al., 1997). A Northern blot analysis of total RNA isolated from induced SCC15TEVp53-19 cells (-Tet), either exposed to IR or mock-irradiated, displayed a significant increase in p21^{WAF1/Cip1} mRNA levels when compared to uninduced SCC15TEVp53-19 cells (Fig. 3). In contrast to what was reported for colorectal cancer cells (Hermeking et al., 1997), no significant (only 1.4 fold) increase of 14-3-3 σ mRNA was observed after induction of wt p53 in the SCC15TEVp53-19 keratinocytes (Fig. 3). In order to make sure that the absence of 14-3-3 σ induction is not due to too high levels of wt p53, we also analyzed the effect of intermediate levels of p53. When reducing p53 gene expression levels by adding increasing concentrations of tetracycline to the medium, the levels of p21^{WAF1/Cip1} mRNA followed the levels of p53 mRNA and decreased in a similar way (Fig. 5). However, also under these conditions, no significant change in the expression of 14-3-3 σ was observed (Fig. 5).

14-3-3 σ is one of seven highly homologous 14-3-3 genes expressed in human cells (van Hemert et al., 2001; Yaffe, 2002). To exclude the possibility that the results obtained with Northern blot analysis are due to cross-hybridization of the 14-3-3 σ probe with mRNA from other genes, the results of the Northern blot analysis were verified by RT-PCR analysis using a unique primerset for 14-3-3 σ (Fig. 6). Whereas RT-PCR analysis showed an increase in the p21^{WAF1/Cip1} level of RNA isolated from p53 expressing SCC15TEVp53-19 cells, no significant change in the expression level of 14-3-3 σ was observed, neither in cells exposed to 10 Gy of IR nor in mock-irradiated cells (Fig. 6). RNA from fibroblasts, which do not express 14-3-3 σ , was included as a negative control and the identity of the 14-3-3 σ RT-PCR products, obtained from the transgenic SCC15 cells, was confirmed by DNA sequencing.

Apparently, 14-3-3 σ is constitutively expressed in SCC15 cells but nevertheless, an efficient G2/M cell cycle arrest was only observed in the p53-expressing SCC15TEVp53-19 cells following exposure to IR, suggesting that, if 14-3-3 σ is involved in G2/M arrest in keratinocytes, it might be regulated at the protein level rather than at the mRNA level. Protein levels of both 14-3-3 σ and p21^{WAF1/Cip1} can be regulated by the ubiquitin-proteasome pathway (Cayrol & Ducommun, 1998; Urano et al., 2002).

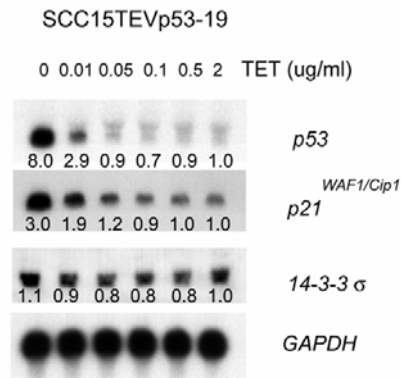


Figure 5. Impact of p53 expression levels on p21^{WAF1/Cip1} and 14-3-3σ mRNA expression: RNA was isolated from SCC15TEVp53-19 or SCC15TEVLuc-1 cultures grown for 2 days in the presence of the indicated amounts of tetracycline. The indicated relative expression levels were normalized for GAPDH (internal control).

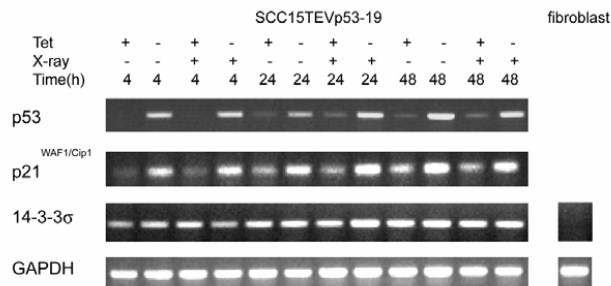


Figure 6. RT-PCR analysis of Tet-regulated p53, p21^{WAF1/Cip1}, 14-3-3σ and GAPDH (control) mRNA levels in SCC15TEVp53-19 cells exposed to 10 Gy of IR or mock irradiated. Cells were cultured in -Tet medium and subsequently changed to +Tet or kept in -Tet medium. Irradiation (when indicated) was performed 24 hours later and at the indicated time points hereafter RNA was isolated. Human fibroblast RNA was used as a negative control for 14-3-3σ expression.

In figure 7 we have analyzed possible changes in 14-3-3σ (and p21^{WAF1/Cip1}) protein levels after p53 induction and/or treatment with ionizing radiation by immunoblotting, using an anti-14-3-3σ antibody previously tested for its specificity (van Hemert et al., 2004). This analysis did not show any significant differences in

14-3-3 σ protein levels in induced or uninduced SCC15TEVp53-19 cells, either exposed to 10 Gy of IR or not irradiated. Protein levels of p21^{WAF1/Cip1}, on the contrary, correlated with the expression of p53 (fig. 7A).

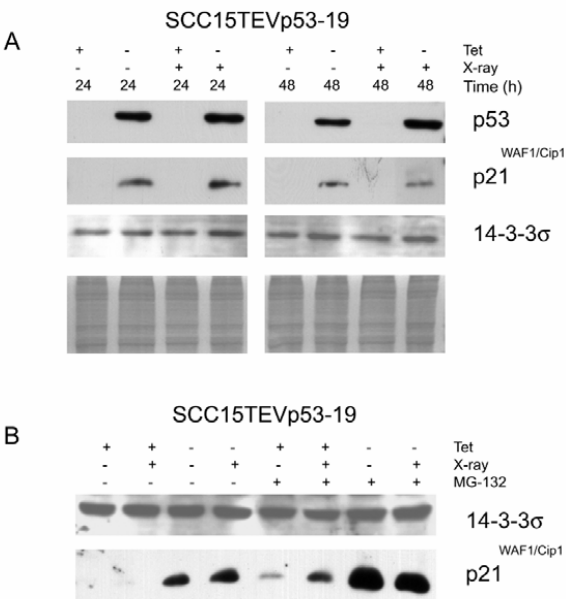


Figure 7. Immunoblot analysis of (A) Tet-regulated p53, p21^{WAF1/Cip1} and 14-3-3 σ protein expression in SCC15TEVp53-19 cells exposed to 10 Gy of IR or mock irradiated. Cells were cultured in -Tet medium and subsequently either changed to +Tet or kept in -Tet medium. Irradiation (when indicated) was performed 24 hours later and at the indicated time points hereafter whole-cell protein extracts were prepared and analyzed by immunoblotting. As a loading control, a section of the gel was stained with Coomassie Brilliant Blue (CBB). (B) Effect of the MG132 proteasome inhibitor on p21^{WAF1/Cip1} and 14-3-3 σ protein levels in SCC15TEVp53-19 cells following (mock-) irradiation in either +Tet or -Tet culture medium. Cells grown for 24 hours in +Tet or -Tet medium were irradiated with 10 Gy of IR (or mock irradiated) and treated 20 h hereafter with 20 μ M of MG132 (in DMSO) or mock-treated with DMSO. 4 h later whole-cell extracts were prepared and analyzed by Western blotting.

In order to exclude a possible involvement of the ubiquitin-proteasome pathway on 14-3-3 σ protein levels in SCC15TEVp53-19 we treated cells with the proteasome inhibitor MG132. Protein levels of p21^{WAF1/Cip1} were again analyzed as a control. A strong increase in p21^{WAF1/Cip1} protein was observed in SCC15TEVp53-19 cells

expressing p53 after proteasome inhibition by MG132 as compared to mock (DMSO) treated cells (fig 7B). Even uninduced SCC15TEVp53-19 cells show a visible p21^{WAF1/Cip1} band after MG132 treatment, which is more prominent in irradiated cells, indicating p53-independent low level induction of p21^{WAF1/Cip1} following irradiation. In contrast, induced, uninduced, irradiated and unirradiated MG132 treated SCC15TEVp53-19 cells all showed similar levels of 14-3-3 σ protein as compared to mock treated cells (fig. 7B), indicating that in these cells the constitutive levels of 14-3-3 σ have overcome proteasome dependent regulation.

Discussion

We have introduced a tetracycline-regulated wt p53 gene into SCC15 cells that lack endogenous p53, and examined the effect of wt p53 expression and/or ionizing radiation on proliferation, cell cycle regulation and on the conditional expression of the p53 target genes, 14-3-3 σ and p21^{WAF1/Cip1}. Increased levels of p53 mRNA and protein were detected in several SCC15 clones, following withdrawal of tetracycline. From the various p53-expressing SCC15 clones that were isolated, only two clones, SCC15TEVp53-19 and SCC15TEVp53-46, that had undetectable levels of p53 mRNA in the presence of tetracycline, expressed wild-type p53 following tetracycline withdrawal, as was concluded from sequencing analysis. Expression of this wild-type p53 transgene resulted in increased p21^{WAF1/Cip1} gene expression and reduced cell growth. In SCC15TEVp53-11, the p53 transgene was mutated which resulted in substitution of a glutamic acid by a lysine residue at position 286.

Residue 286 is located in the DNA binding domain, the region where more than 90% of all p53 mutations in cancer are found. Substitution of E286 was previously detected in several types of cancer (Feki & Irminger-Finger, 2004) and it was established that a substitution of E286 constitutes a loss-of-function mutation which results in a complete loss of the transactivation capacity (Thukral et al., 1994). Our finding that expression of a p53 transgene with an E286 substitution in SCC15TEVp53-11 cells did not result in any measurable increase of p21^{WAF1/Cip1} and did not affect the growth properties of SCC15 cells further highlights the functional

dependence between expression of wild-type p53 expression, p21^{WAF1/Cip1} transactivation and the cellular consequences that were observed.

In the EJ human bladder carcinoma cell line conditional expression of wild-type p53 resulted in irreversible growth arrest in G1 and G2/M, reflecting features of cellular senescence (Sugrue et al., 1997). A similar outcome was observed after conditional expression of p21^{WAF1/Cip1} in the same cells (Fang et al., 1999). Analysis of the cell cycle distribution in our SCC15TEVp53-19 keratinocyte culture revealed that wild-type p53-expressing SCC15 cells were mainly arrested in the G1/S but not in the G2/M phase of the cell cycle. Although induced SCC15TEVp53-19 cells displayed some features of replicative senescence (e.g. increased cell size and a flatter appearance, Fig. 1D), growth curve analysis indicated that the G1/S cell cycle arrest monitored by flow cytometric analysis was transient, since the cells continued to proliferate albeit at a reduced rate. Since this reduced rate of cell proliferation in p53 expressing SCC15 cells occurred despite high levels of p21^{WAF1/Cip1} transcription (Fig. 3), it follows that p21^{WAF1/Cip1} is not able to impose a similar prominent cell cycle arrest in our cell system as in the bladder carcinoma cell lines discussed above. This difference is likely inherent to the use of a keratinocyte background. It has been shown by others that high levels of p21^{WAF1/Cip1} inhibit late stages of keratinocyte terminal differentiation (Di Cunto et al., 1998). The down-modulation by proteolytic degradation of p21^{WAF1/Cip1} that occurs at late stages of keratinocyte differentiation could underlie the less prominent cell cycle arrest that we have observed. Our finding, that a major part of the p53-induced p21^{WAF1/Cip1} protein is indeed rapidly degraded by the proteasome system in SCC15 cells (Fig. 7), is in line with this vision.

Following exposure to ionizing radiation a clear shift from a G1/S to a G2/M cell cycle arrest was observed in SCC15 cells expressing wt p53. These observations are in line with the findings of Flatt *et al.* (Flatt et al., 1998), who have studied the effect of IR on normal human keratinocytes and have also monitored a predominant G2/M cell cycle arrest. Our results show that in keratinocytes a combination of both ionizing radiation and expression of wt p53 is required to induce an efficient G2/M arrest. This increase in G2/M is accompanied by a clear attenuation of the G1/S cell cycle arrest (Fig. 4). A similar attenuation has also been observed in normal human keratinocytes following IR by Flatt and coworkers (Flatt et al., 1998). In contrast to

fibroblasts, where IR results in a complete inhibition of DNA synthesis, human keratinocytes continue to cycle at reduced rate and accumulate in G2/M (Flatt et al., 1998). Our results indicate that X-ray irradiation was not able to further increase either p21^{WAF1/Cip1} transcript (Fig. 6) or protein levels (Fig. 7) in cells expressing wild-type p53. As these levels are not sufficient for permanent arrest in G1/S (Fig. 1 and discussion above), the irradiated cells will progress in the cell cycle until the G2/M boundary, where they will arrest. This can be concluded from the p53-dependent increase in G2/M phase and the decrease in S-phase both after 6 and 10 Gy X-ray exposure (Fig. 4B/C). Such a decrease in S-phase cells is not observed in irradiated cells not expressing p53 (Fig. 4A/B, +Tet) or cells expressing mutant p53 (Fig. 4D), indicating that in keratinocytes a prominent radiation induced G2/M arrest is depending on wt p53 expression.

As we have shown that in keratinocytes G2/M cell cycle arrest is both p53 and IR dependent it was interesting to look for p53 target genes that would be responsible for this outcome. An obvious candidate for such a function is 14-3-3 σ (stratifin), which was previously shown to play an important role in p53-dependent G2/M cell cycle arrest after DNA damage (Hermeking et al., 1997) by sequestering the cdc2-cyclinB1 complex in the cytoplasm (Chan et al., 1999). The involvement of 14-3-3 σ in G2/M cell cycle arrest is often assumed to be a general property of this protein. However, experiments that show a direct link between p53 expression, 14-3-3 σ induction and G2/M cell cycle arrest were performed almost exclusively in colorectal cancer cell lines. In contrast to the negative role of 14-3-3 σ in cell proliferation in human colorectal cancer cells after DNA damage, induction of 14-3-3 σ appears to play a positive role in IGF-I receptor-induced cell proliferation in MCF-7 breast cancer cells (Zhang et al., 2004) or in androgen receptor and epidermal growth factor signaling in prostate cells (Huang et al., 2004). In both cell types this function of 14-3-3 σ was p53-independent.

In our keratinocyte background we did not observe significant changes in 14-3-3 σ transcript or protein levels following wild-type p53 expression or after irradiation with ionizing radiation (Figs. 3, 5, 6 and 7). Instead, in the SCC15 cells 14-3-3 σ appears to be constitutively expressed, as it is in normal keratinocytes (Dellambra et

al., 2000). Apparently, the biological function of 14-3-3 σ in keratinocytes is independent of p53 cell cycle control.

In human keratinocytes 14-3-3 σ is required for clonal evolution, that is the transition from stem cells to transit-amplifying cells. Indeed, Dellambra and co-workers have shown that down-regulation of 14-3-3 σ forces primary keratinocytes into the stem cell compartment (Dellambra et al., 2000), prevents replicative senescence and promotes immortalization of primary human keratinocytes. As transit-amplifying cells are still able to proliferate, although with a lower capacity for self-renewal and a higher probability of undergoing terminal differentiation, it is indeed unlikely that 14-3-3 σ expression would result in a permanent cell cycle arrest in keratinocytes as it does in colorectal cancer cells (Hermeking et al., 1997). Similarly, the skin of keratin 10 null mice displays hyperproliferation of basal cells despite highly increased expression levels of 14-3-3 σ mRNA and protein (Reichelt & Magin, 2002) and in several hyperproliferative human skin diseases high levels of 14-3-3 σ have been recorded.

In conclusion, we have generated cell lines that allow investigation on the influence external factors like ionizing radiation, either in the presence or absence of wild-type p53, in an otherwise genetically identical human keratinocyte background. We have shown that expression of wild-type p53 results in decreased cell proliferation due to a transient G1/S cell cycle arrest. After ionizing radiation a clear shift from a transient G1/S to a more prominent G2/M cell cycle arrest was observed. This G2/M arrest depended largely on the expression of wild-type p53, but was not mediated by increased expression or stabilization of the 14-3-3 σ protein. In keratinocytes the biological function of 14-3-3 σ appears to reside above all in the control of long-term processes such as cell senescence and/or terminal differentiation (Dellambra et al., 2000) rather than in the acute inhibition of cell cycle progression after DNA damage.

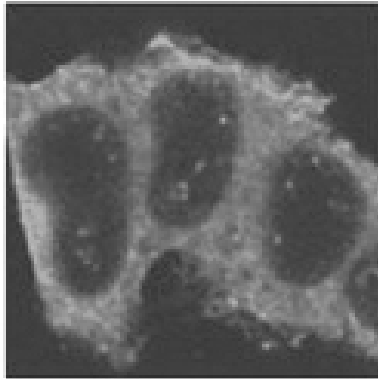
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Chapter 6



TwinGFP, a marker for cell cycle analysis in transiently transfected cells

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Abstract

Green fluorescent protein is widely used as a marker to identify transfected cells either by fluorescence microscopy or flow cytometry. However, cell cycle analysis with propidium iodide typically employs ethanol for cell permeabilization. During this treatment soluble GFP proteins generally leak out of cells, probably due to their small size. We have now significantly improved cellular retention by creating an in-frame fusion of two GFP DNA sequences, thus generating a double-sized GFP protein (TwinGFP: 57 kD). Permeabilized HeLa cells transfected with pTwinGFP showed a strong green fluorescent signal localized throughout the cells, which could easily be detected by fluorescence microscopy and flow cytometry in contrast to cells transfected with a standard single GFP construct. The experiment indicates that protein size constitutes the major determinant for the loss of fluorescence in permeabilized cells. As a proof of principle, pTwinGFP was co-transfected with the p53 tumor-suppressor gene into HeLa cells, and cells transiently expressing p53 could be identified and phenotypically characterized by flow cytometry.

Keywords: GFP duplication; green fluorescent protein; p53; cell permeabilization; apoptosis; FACS

Introduction

Cell-cycle arrest and apoptosis are routinely studied by flow cytometry (Ormerod, 2002). By definition, sustained expression of gene-products inducing these regulated cellular processes is not possible, which eliminates the possibility to create stable viable cell lines. Conditional expression of this class of genes is a possibility but the approach is very labor-intensive and the probability to select mutants is very high (Niemantsverdriet et al., 2005). Transient transfections constitute an interesting alternative but are often hampered by the low percentage of cells that take up DNA during most transfection methods.

Enhanced green fluorescent (GFP) protein is widely used as transfection marker because its fluorescence is independent of other proteins, co-factors or substrates and it is expressed as an inert soluble protein throughout the cell (Schmitz & Bereiter-Hahn, 2001; Tsien, 1998). Plasmids expressing GFP proteins can be co-transfected in combination with plasmids expressing proteins involved in cell-cycle arrest or apoptosis to identify those cells that have taken up the gene of interest. However, protocols for cell-cycle progression or apoptosis studies by flow cytometry rely on effective and quantitative measurement of the DNA content by propidium iodide staining and require permeabilization of the cells, which is routinely achieved with ethanol treatment (Kalejta et al., 1999; Kalejta et al., 1997). Observations in a number of laboratories have indicated that the commercially available GFP proteins are not retained in the cells after ethanol treatment (Kalejta et al., 1999; Kalejta et al., 1997; Kusser & Randall, 2003). To prevent leakage of GFP, cells have been fixed with cross-linking agents prior to permeabilization. Several disadvantages have, however, been observed using this approach: GFP associated nonspecifically with microfilaments (Schmitz & Bereiter-Hahn, 2001), its conformation changed resulting in a decrease of brightness (Kusser & Randall, 2003), the cell morphology was affected and fixation has also been found to interfere with detection by flow cytometry (Kalejta et al., 1997; Kusser & Randall, 2003). Other researchers have tagged their GFP with targeting signals that direct the protein to specific cellular compartments or induce membrane-anchorage. These adaptations have indeed

resulted in the retention of GFP in permeabilized cells (Harvey et al., 2001; Kalejta et al., 1999; Kalejta et al., 1997).

If the loss of GFP from permeabilized cells is simply due to its small molecular size, then a double-sized GFP protein, created by recombinant gene-duplication, which can be expected to have the same properties as the single protein, should provide a useful complement to targeted GFP proteins. Here we show that TwinGFP has indeed the same cellular localization as the single protein but it is retained in ethanol treated cells. Experimental data show that TwinGFP can be used to identify and gate transiently transfected cells in flow cytometry, even at low transfection efficiency.

Materials and methods

Plasmids

pTwinGFP was constructed by ligating the fragment encoding EGFP from pEGFP-C1 (Clontech Inc., Palo Alto, CA) digested with SalI and Eco47-III in the pEGFP-N1 plasmid (Clontech Inc., Palo Alto, CA) digested with XhoI and Eco47-III. Upon expression the resulting fusion consists of two identical EGFP sequences separated by a 34 amino acid spacer, derived from parts of the multiple cloning site sequences (GLRSRAQASNSAVELKLRILQSTVPRARDPPVAT). pCMV-Neo and pC53-SN3 (Baker et al., 1990) were generously provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). Plasmid pcDNAp53 was constructed by inserting a DNA fragment containing the complete p53 coding sequence from pC53-SN3 into the BamHI site of pcDNA3 (purchased from Invitrogen) in the correct orientation.

Cell culture, transfection and microscopy

HeLa and Saos-2 cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum. HeLa cells were grown to 70% confluence on 6 cm dishes, washed twice with DMEM and transfected with a total of 5 µg plasmid DNA per dish using the liposome transfection reagent DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*, *N*, *N*-trimethylammonium methyl-sulfate) (Roche). Saos-2 cells were transfected by using

the Amaxa system according to the recommendations of the supplier (Amaxa Biosystems). Transfected cells were analyzed with a Zeiss Axiovert 135 microscope, equipped for GFP visualization with a DAPI/GFP filter (Zeiss) and for propidium iodide plus GFP visualization with a triple-band CFP/YFP/dsRed filter (Omega Optical Inc.). The fluorescence of microscopic pictures was quantified by using the imageJ (NIH, Bethesda) freeware program.

Flow cytometry

Cells were trypsinized, suspended in PBS containing 10% serum (for trypsin inactivation), washed with PBS and resuspended in 300 μ l PBS, permeabilized by adding 700 μ l cold (-20°C) 100% ethanol and samples were kept at -20°C . Before analysis with a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), cells were pelleted, resuspended in 0.2 ml PBS and treated with RNase A (10 $\mu\text{g/ml}$) and propidium iodide (10 $\mu\text{g/ml}$ for microscopic analysis and 100 $\mu\text{g/ml}$ for FACS analysis) for 30 min. at room temperature. For each sample 100.000 cells were sorted and analyzed using WinMDI 2.8 software.

Results

Because the basic properties of EGFP are very suitable for flow cytometry measurements and the protein size might be the major problem when permeabilization is required, we constructed pTwinGFP, a plasmid that expresses a double sized EGFP protein (Fig. 1 A, see materials and methods for details). After transfection of the resulting pTwinGFP plasmid into HeLa cells, green fluorescence was readily observed throughout the cells (Fig. 1 B). Phase contrast visualization of the same microscopic field showed no differences in morphology between cells expressing TwinGFP protein and untransfected cells (Fig. 1 C).

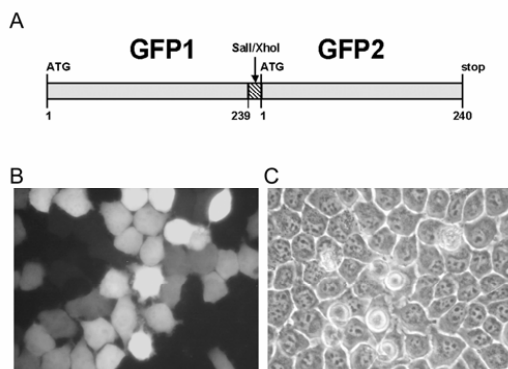


Figure 1. Cellular distribution of TwinGFP. **(A)** Schematic representation of the TwinGFP construct. Two identical GFP sequences (GFP1 and GFP2) are separated by a spacer sequence (hatched). Start codons of both GFP sequences (ATG), the stop codon (second GFP), the restriction enzymes used for the construction and the amino acid numbering of the two individual GFP sequences are indicated. **(B)** Fluorescence analysis of HeLa cells, 24 hours after transfection with pTwinGFP. **(C)** Phase contrast visualization of the same microscopic field as in (B).

The intensity and cellular distribution of the fluorescence of TwinGFP was very similar to the one found after transfection of the same cells with either pEGFP-C1 or pEGFP-N1 (Fig. 2 B). As expected, no fluorescence was observed in HeLa cells transfected with the control pCMV-Neo vector (Fig. 2 B). Immunoblot analysis of protein extracts derived from pTwinGFP transfected HeLa cells showed that indeed a double sized GFP protein was expressed (data not shown). When HeLa cells transfected with pTwinGFP were trypsinized and treated with ethanol, green fluorescence was clearly visible, in contrast to cells transfected with pEGFP-C1 or pEGFP-N1 (Fig. 2 C). Counterstaining DNA with propidium iodide (PI) (red fluorescence) indicated that all cells were indeed permeabilized (Fig. 2 D). After permeabilization of pTwinGFP transfected cells, a consistent decrease (approximately 30%) in the number of fluorescent cells was found (compare the TwinGFP panels in Fig. 2B and 2C), indicating that duplication of the GFP reading frame significantly diminishes cellular leakage of the protein, but does not prevent it completely.

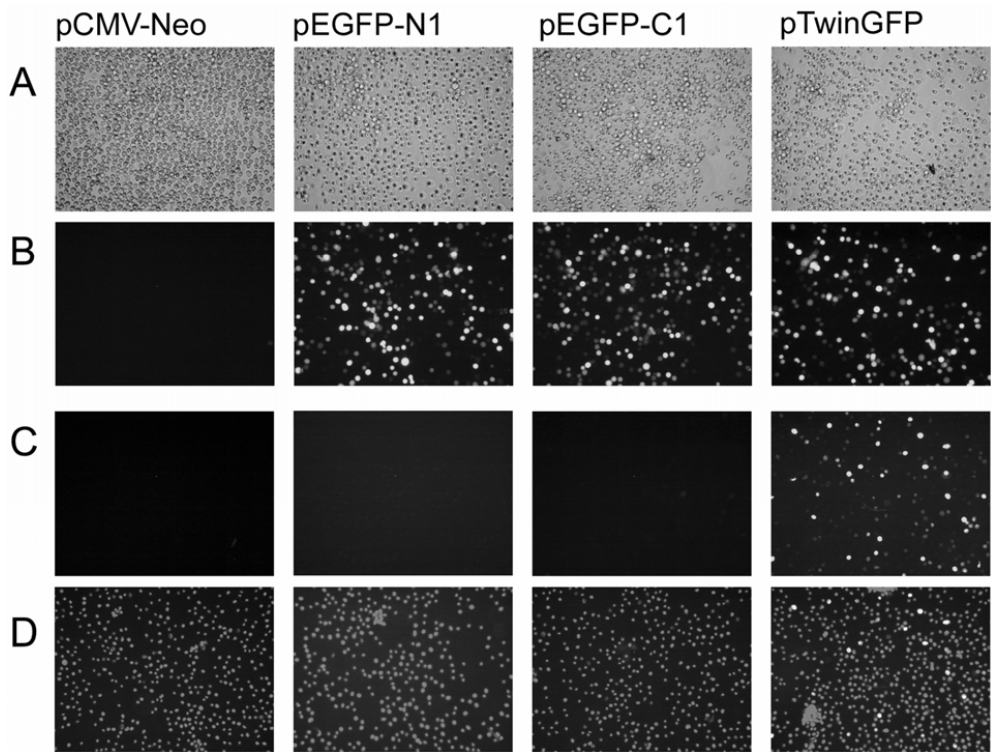


Figure 2. TwinGFP is retained in transfected cells after ethanol treatment. HeLa cells were transiently transfected with pCMV-Neo, pEGFP-N1, pEGFP-C1 or pTwinGFP. After 24 hours cells were trypsinized, suspended in PBS containing 10% serum and one part of the cell suspension was immediately photographed either under phase contrast illumination **(A)** or fluorescence visualization using a DAPI/GFP filter **(B)**. A second part of the cell suspension was ethanol-treated at -20°C , suspended in PBS containing RNase A and propidium iodide (see Materials & Methods) and visualized by fluorescence microscopy, using either a DAPI/GFP for GFP detection **(C)** or a triple band CFP/YFP/dsRed filter for both GFP (green) and propidium iodide (red) detection **(D)**.

Besides HeLa cells (cervix adenocarcinoma), we have also tested Saos-2 cells (osteosarcoma) for improved retention of TwinGFP after EtOH permeabilization. The results are very similar to the ones described for the HeLa cells and indicate that also in Saos-2 cells a substantial amount of (60-65%) of GFP transfected cells retain green fluorescence after EtOH permeabilization, in contrast to pEGFP-N1 and pEGFP-C1 transfected cells (Fig. 3).

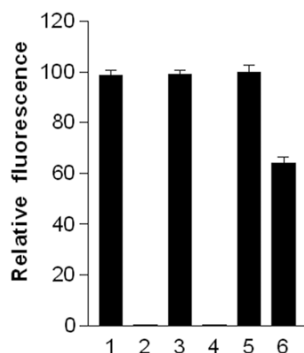


Figure 3. TwinGFP is retained in ethanol permeabilized Saos-2 cells. Saos-2 cells were transfected with pEGFP-N1 (bars 1 & 2), pEGFP-C1 (bars 3 & 4) or pTwinGFP (bars 5 & 6) treated and analyzed 24 hours later by fluorescence microscopy as described in the legend of figure 2. Cells were trypsinized and analyzed immediately (bars 1, 3, 5) or permeabilized by ethanol treatment as described in the legend of figure 2 (bars 2, 4, 6). The relative green fluorescence (per cell) was determined by using the ImageJ software. The relative fluorescence of the non-permeabilized pEGFP-N1 and pEGFP-C1 (bars 1 & 3) was set at 100%.

Having established that the TwinGFP protein is retained efficiently in various cell types after ethanol treatment, we analyzed the cell cycle distribution of transfected cells by flow cytometry. While cells transfected with pCMV-Neo showed a dot-plot pattern that resembled the pattern of untransfected cells (Fig. 4 A, B, left panels), cells transfected with pTwinGFP also showed a cell population (approximately 38%) with measurable GFP fluorescence levels (Fig. 4 A, B, right panels). No effects of TwinGFP on the cell cycle were observed (Fig. 4C, compare left and right panels).

Expression of p53 has been used previously to show the functionality of plasmids expressing GFP proteins modified for flow cytometric purposes (Kalejta et al., 1997). P53 is a tumor suppressor gene mutated in many human tumors. When overexpressed as the wild-type protein in cultured cells, it induces cell cycle arrest or apoptosis (Vogelstein et al., 2000). Apoptotic cells are characterized by extensive DNA degradation, which can be identified as a pre-G1 peak in DNA histograms obtained by flow-cytometry. (Haupt et al., 1995; Iwashina et al., 1998; Ormerod, 2002). It was established that HeLa cells transiently transfected with a p53 expression plasmid show a pre-G1 peak indicating apoptosis (Haupt et al., 1995).

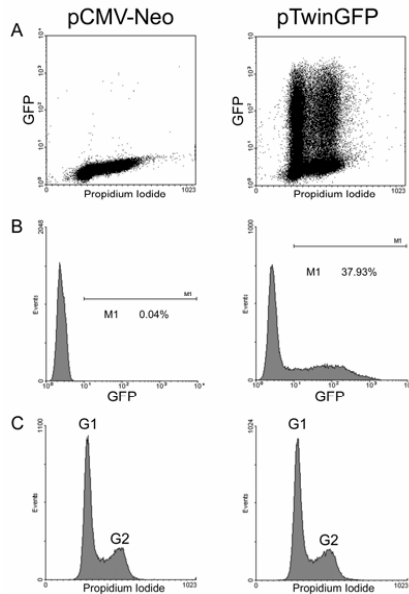


Figure 4. TwinGFP can be detected by flow cytometry in ethanol-treated cells. HeLa cells were transfected with pCMV-Neo (left column) or pTwinGFP (right column), 24 hours after transfection cells were treated with ethanol and DNA was stained with propidium iodide before flow cytometric analysis. **(A)** Bivariate dot-plots showing the distribution of green fluorescence on the y-axis (GFP) versus the red fluorescence of propidium iodide staining (representing DNA content) on the x-axis. **(B)** Histogram of the distribution of green fluorescence along the y-axis of the respective dot-plots in panel A; M1 represents the fraction of cells showing a GFP signal. **(C)** DNA histograms (red fluorescence) corresponding to the cells analyzed in the respective dot-plots in panel A. G1 and G2 phases of the cell cycle are indicated.

We co-transfected 4 μ g pcDNAp53 (pcDNA3 expressing wild type p53) or pcDNA3 (empty vector control) (Baker et al., 1990) with 1 μ g pTwinGFP into HeLa cells. 24 hours later, cells were trypsinized, treated with ethanol and the DNA was stained with propidium iodide. In a control experiment (Fig. 5 A, B, C), where HeLa cells were co-transfected with pcDNA3 and pTwinGFP, less than 5% of the cells showed a pre-G1 population. This value was not significantly influenced by gating cells with a low (area R1) or a high (area R2) GFP signal (Fig. 5 B, C, G, open bars). Similar background levels were previously found by others in HeLa cells (Haupt et al., 1995). Cells expressing p53 (Fig. 5 D, E, F) showed a clear increase in apoptotic cells which was directly related to the GFP signal. In the population with a low GFP signal,

indicative of low p53 expression (area R1), approximately 8% of the cells were apoptotic (Fig. 5 E, G, closed bar). In the population with a high GFP signal (area R2) the percentage of apoptotic cells increased until 18.0% (Fig. 5 F, G, closed bar).

Discussion

Because GFP proteins are not retained in ethanol treated cells, we constructed plasmid pTwinGFP by creating an in-frame fusion of two EGFP DNA sequences. After ethanol treatment, both HeLa and Saos-2 cells transfected with pTwinGFP showed a clear green fluorescence, which could be detected by fluorescence microscopy and quantified by flow cytometry. No fluorescence was observed in permeabilized cells transfected with plasmids expressing single EGFP. Although some decrease in the number of fluorescent cells was observed in both cell lines, 60-70% of the cells are still clearly positive, indicating that protein size is the major reason for the loss of this protein from ethanol treated cells. Apparently by increasing the size of GFP, the twin protein is less likely to diffuse out of permeabilized cells. However, as ethanol is also a mild fixative, it can not be completely ruled out that TwinGFP is more easily immobilized than its single counterpart. It can also not be ruled out that other factors might be responsible for the difference in functionality in cytometric experiments between single GFP proteins and TwinGFP. In any case, our research demonstrates that a significant amount of TwinGFP is retained in ethanol treated cells and constitutes as such a useful innovation for cytometric studies. Hence, TwinGFP can be used as a marker to identify cells transiently co-transfected with a given gene of interest. As the focus in this type of experiments is generally on the identification of the transfected rather than the untransfected cells (which can be studied in a control transfection where the gene of interest has been omitted), the loss of some of the fluorescence during the permeabilization step (see above) should not constitute a major obstacle. That this is indeed the case was shown by the p53 co-transfection experiment.

When pTwinGFP was co-transfected with a plasmid expressing p53, an increase in apoptotic cells was measured compared to pcDNA3 transfected control

cells. More importantly, the percentage of apoptotic cells was higher in cells showing a high GFP signal than in cells showing a low GFP signal, indicating a direct correlation between the expression level of TwinGFP and the expression level of the gene of interest (p53 in our case). This makes TwinGFP a suitable transfection marker. The percentage of cells that take up plasmid DNA during transfection varies and both transfection method and cell type influence the transfection efficiency. By using TwinGFP as a transfection marker in co-transfection experiments, flow cytometric measurements can be corrected for low transfection efficiency by sorting a large number of cells and selecting those cells that have taken up high amounts of plasmid DNA by gating the cells with high green fluorescence.

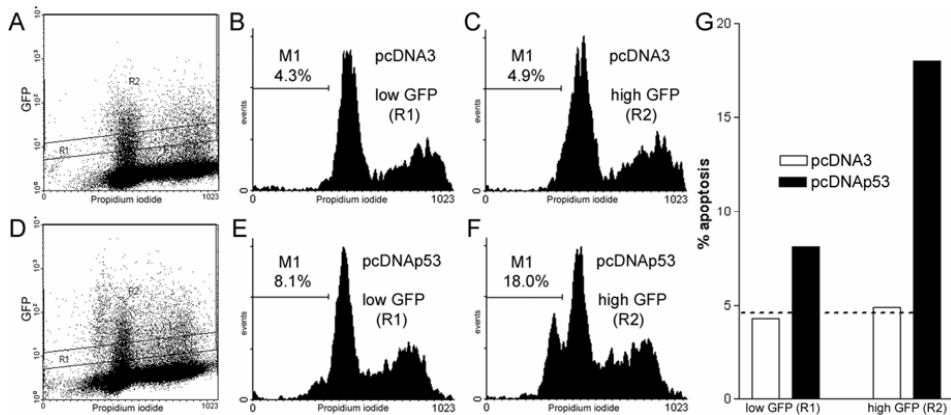


Figure 5. TwinGFP fluorescence reflects the expression level of a co-transfected gene (p53). HeLa cells were co-transfected with either 4 μ g pcDNA3 (A, B, C) or pcDNAp53 (D, E, F) and 1 μ g pTwinGFP; 24 hours after transfection, both adherent and non-adherent cells were treated with ethanol and DNA was stained with propidium iodide before flow cytometric analysis. **(A, D)** Bivariate dot plots representing GFP fluorescence versus DNA content; the gated cell populations R1 (low GFP content) and R2 (high GFP content) are indicated. **(B, E)** DNA histograms of low green fluorescent cells (gate R1 in panels A and D respectively); M1 represents the fraction of apoptotic cells (pre-G1 population). **(C, F)** Cell cycle distribution of high green fluorescent cells (gate R2); M1 indicates the fraction of apoptotic cells (pre-G1 population). Note the extra pre-G1 peak in panel F. **(G)** Direct graphic comparison of apoptotic cell fractions in the gated R1 (low GFP) and R2 (high GFP) cell populations after transfection of either wild-type p53 (pcDNAp53) (black bars) or a control plasmid (pcDNA3) (white bars). Results are derived from 3 independent experiments. The average background level of apoptosis in the HeLa cell cultures (after transfection) is indicated by a dotted line.

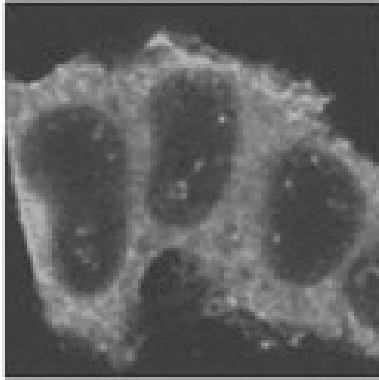
Acknowledgements

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Chapter 7



RT-PCR analysis of p73 splice variants, ease or tease?

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Maarten Niemantsverdriet, Wilbert P. Vermeij and Claude Backendorf

TO THE EDITOR

The gene TP73 has been identified as a homologue of tumor suppressor TP53 and is expressed from 1p36, a region frequently deleted in human cancers (Melino et al., 2002). TP73 is expressed from two distinct promoters, which results in TAp73 and Δ Np73 isoforms. TAp73 isoforms contain a transactivation domain and are able to act as transcription factors and transactivate p53 target genes like p21WAF1/Cip1 and 14-3-3 σ . Δ Np73 isoforms lack the transactivation domain and can inhibit the function of TAp73 isoforms and p53. TP73 also presents a wide array of C-terminal splice variants called α , β , γ , δ , ϵ and ζ . The resulting p73 proteins have different biological functions and are involved in development, apoptosis, cell-cycle arrest and oncogenesis. Several types of leukemia and solid tumors express much higher levels and alternative combinations of p73 splice variants compared to the tissue from which they originated (Melino et al., 2002; Sahu et al., 2005). Hence, the identification of all possible splice variants is essential to fully understand the involvement of TP73 in carcinogenic transformation. However, contrasting results regarding p73 mRNA expression in haematopoietic malignancies obtained by different investigators have been noted (Sahu et al., 2005).

RT-PCR is a simple and easy method that has been frequently used to identify C-terminal p73 splice variants expressed in various human cell types and tissues. By choosing PCR primers outside the region spliced out, several p73 forms can be identified simultaneously (Scaruffi et al., 2000; Zwahlen et al., 2000; Ishimoto et al., 2002; Tschan et al., 2000). Based on results obtained by this method specific p73 splice variants have been linked to various forms of leukaemia (Scaruffi et al., 2000; Tschan et al., 2000).

In an effort to further validate this approach, we have tested whether the detection of all C-terminal p73 splice variants expressed in a given tissue is independent of the total amount of cDNA analysed. For this purpose, we have used four different concentrations of cDNA derived from cultured SCC15 cells (human squamous cell carcinoma) as template and PCR primers in exons 8 and 14 of p73 (Zwahlen et al., 2000).

Unexpectedly, our analysis showed that not all p73 splice variants present in the cDNA sample are detected in every PCR reaction. In the PCR reactions with the two lowest amounts of SCC15 cDNA, splice variants p73 α , p73 γ and p73 ϵ were detected (Figure 1a). In the PCR mixture containing the second highest amount of cDNA, a p73 δ band and a lower (221 bp) band appeared whereas p73 α was hardly visible (Figure 1a), while in the PCR reaction with the highest amount of cDNA the 221 bp band was the only band detected (Figure 1a). The identity of splice variants was determined by sequencing (Figure 1b, data not shown). Since the smallest C-terminal splice variant described in almost all p73 research and review articles is p73 δ (Melino et al., 2002; Ishimoto et al, 2002; Tschan et al., 2000) we focused specifically on the 221 bp band. Sequence analysis showed that the 221 bp band was indeed amplified from a genuine p73 splice variant, where exons 10, 11, 12 and 13 were spliced out (Figure 1b). The same splice variant was described previously as p73 η in this journal by Scaruffi et al. A schematic representation of the p73 splice variants including the p73 η form is presented in Figure 1c. Scaruffi et al. (Scaruffi et al., 2000) have suggested a specific role for p73 η in the differentiation of acute lymphoblastic leukaemia (ALL) based on their finding that the expression of this variant is restricted to a small percentage of more differentiated B-cell ALL (This p73 η splice variant should not be confused for another splice variant first described by Ishimoto et al. (Ishimoto et al, 2002), also denominated p73 η ; this form is characterised by alternative termination, has a long exon 13 containing a stop codon and encodes a 571-amino-acid protein). Besides, its presence in SCC15 cells, the p73 η form was also detected in HaCaT (immortal keratinocyte cell line) and HCT116 (colorectal cancer cell line) cells (Figure 1d). It was not detected in HeLa cells (cervix carcinoma cell line) and in cultured primary human cells (keratinocytes and fibroblasts), despite the use of high concentrations of cDNA in the PCR reaction. Hence, we found expression of p73 η in three out of four transformed cell lines analysed (Figure 1a and d), but detection of p73 η (and other splice variants) depended strongly on the concentration of template used. This indicates that extreme care must be taken when making statements on the absence or presence of p73 splice variants from results obtained by RT-PCR. Our results demonstrate that when shorter splice variants of p73 are present in amplifiable amounts in the PCR

mixture, larger splice variants become underrepresented during amplification (Figure 1a). This PCR bias (Shagin, DA et al. *Nucleic Acids Res* 1999; 27, e23) cannot be circumvented by using more sophisticated PCR techniques such as real-time RT-PCR, which has the same preference towards the amplification of small splice variants as conventional RT-PCR.

In conclusion, we believe that RT-PCR of different splice variants of p73 can be used in a meaningful way for qualitative purposes, provided that care is taken to analyse various dilutions of the template cDNA. For quantitative purposes it will be imperative to design splice-variant specific primers to produce unique PCR fragments that can be quantified by real-time PCR. Only if these precautions are being taken, meaningful statements on possible links between specific p73 splice variants and a specific carcinogenic phenotype can be made.

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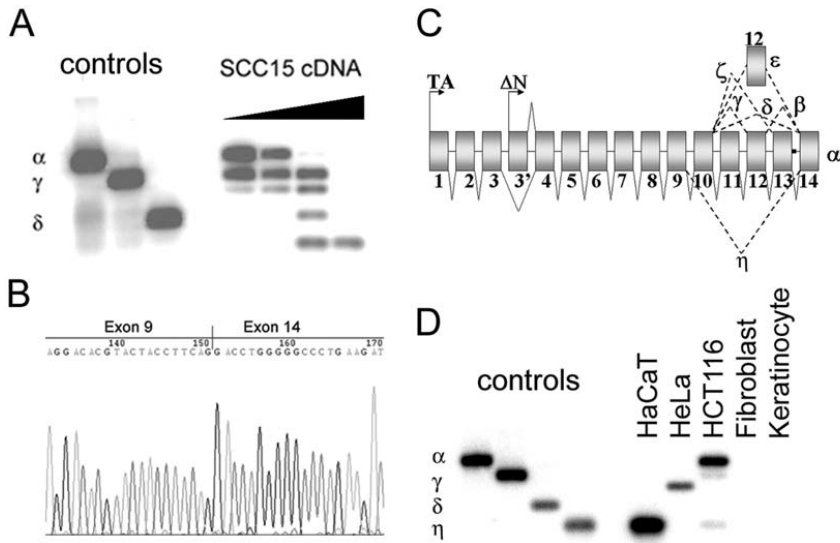


Figure 1. (a) RT-PCR analysis of C-terminal p73 splice variants in SCC15 cDNA. PCR was performed with primers in exons 8 and 14 of p73, (Zwahlen et al., 2000) using 1 ng of plasmids pcDNA3Tap73 α , pcDNA3Tap73 γ , pcDNA3Tap73 δ and increasing amounts (2, 4, 6 or 8 l) of SCC15 cDNA as template. SCC15 cells were cultured at 37°C under 5% CO₂ in DMEM/F12 (ratio 3:1) supplemented with 5% foetal bovine serum (Hyclone) and 0.4 g/ml hydrocortisone (Sigma). RNA was isolated with TRIzol® (Invitrogen) and in RT reactions Super-RT (Sphaero Q) was used as described by the manufacturers. PCR was carried out for 35 cycles (20 s, 94°C; 30 s, 53°C; 1 min, 72°C) after a 10 min 'hot start' at 94°C and followed by final elongation at 72°C for 5 min. The reaction mixture contained 20 pmol of each primer, 1.25 units of AmpliTaqGOLD™ (Roche) and 1.5 mM MgCl₂ in a total volume of 50 l. Control PCR products were run on a 1% agarose gel together with SCC15 cDNA PCR products. DNA was transferred to Hybond-N (Amersham) and hybridised with a p73 specific probe. (b) Sequence analysis of the p73 η splice variant: the 221 bp band (Figure 1a) was amplified, purified and sequenced. A portion of the sequence where exon 9 and 14 are connected is shown. (c) Schematic representation of p73 splice variants, including the p73 η variant. (d) RT-PCR analysis of C-terminal p73 splice variants in different cell types. HaCaT (immortal keratinocyte cell line) and HeLa cells (cervix carcinoma) were cultured in DMEM supplemented with 10% foetal bovine serum. HCT116 cells (colorectal carcinoma) were cultured in RPMI1640 supplemented with 10% foetal bovine serum (Hyclone). Primary human keratinocytes were isolated from foreskin derived from circumcision and grown in serum-free medium (KSFM; Gibco). Primary human fibroblasts were grown in DMEM/F10 in a 1:1 ratio, supplemented with 10% foetal bovine serum (Hyclone). All cells were grown at 37°C under 5% CO₂. The controls for p73 α , p73 γ and p73 δ are the same as in Figure 1a. The control band for p73 η is the 221 bp PCR product of SCC15 cells. The p73 specific probe was made by digestion with AflIII (exon 9) of a PCR product with 5'-GTCACCGGCAGGCAGAGC (exon 6) and 5'-GCTCTCTTTCAGCTTCATCAGG (exon 10) on pcDNA3p73 α (the largest fragment was used as probe).

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Samenvatting voor de leek

De cel is de kleinste eenheid levend materiaal van het menselijk lichaam. Cellen zijn normaal gesproken zo klein dat ze afzonderlijk alleen met een microscoop te zien zijn. Omdat er veel verschillende organen in een menselijk lichaam aanwezig zijn, zijn er ook veel verschillende typen cellen. Zo bestaat bijvoorbeeld de lever uit andere cellen dan de dikke darm en die bestaat weer uit andere cellen dan de huid. Er is dus een grote verscheidenheid aan cellen en die cellen vormen verschillende weefsels. Ieder type cel vervult een bepaalde functie die meer of minder gespecialiseerd is. Dit komt doordat er in de verschillende celtypen verschillende eiwitten aangemaakt worden.

Eiwitten (ook wel proteïnen genoemd) zijn de “werkpaarden” van cellen. Er zijn eiwitten die in alle celtypen nodig zijn, bijvoorbeeld om ervoor te zorgen dat de cellen kunnen delen, dat ze blijven leven etc. Ook zijn er eiwitten die alleen nodig zijn om bepaalde specifieke functies van een cel uit te voeren, deze komen alleen voor in de typen cellen waar ze nodig zijn. Zo zijn er eiwitten die bijvoorbeeld wel in levercellen voorkomen maar niet in huidcellen en vice versa. Eiwitten worden gemaakt in de cellen naar aanleiding van een soort beschrijving die vastgelegd ligt in het DNA. Het “alfabet” die de beschrijvingen vormen bestaat uit maar vier letters, A, C, G en T (basen). Een bericht noemt men een gen. In een cel worden constant verschillende signalen gegeven die ervoor zorgen dat de eiwitten die op dat moment in dat celtype nodig zijn gemaakt worden. Dit gebeurt dus volgens de beschrijving die in het DNA is vastgelegd.

Er zijn nog enkele tussenstappen nodig om van DNA naar eiwit te gaan. Omdat het DNA intact moet blijven zodat er steeds opnieuw de noodzakelijke eiwitten aangemaakt kunnen worden, wordt er als tussenvorm (tussen DNA en eiwit) RNA gemaakt. RNA is vergelijkbaar met DNA en bestaat ook uit 4 letters A, C, G en U (in plaats van T) maar in tegenstelling tot DNA dat de informatie bevat om alle eiwitten te maken, bevat het RNA alleen de informatie die nodig is om één eiwit te maken. Als er veel van hetzelfde eiwit nodig is wordt er ook veel RNA van gemaakt.

Het RNA is maar tijdelijk nodig (totdat het eiwit gemaakt is) en het is dus ook niet belangrijk voor een cel dat het intact blijft. Als er meer van nodig is kan er altijd meer gemaakt worden naar aanleiding van de code in het DNA.

Een gen bestaat naast delen die nodig zijn om een eiwit te beschrijven (exons) uit delen die niet gebruikt worden voor het eiwit (introns). Uit het RNA worden de introns verwijderd en de exons aan elkaar geplakt. Van sommige genen kunnen meerdere eiwitten gemaakt worden die enigszins van elkaar verschillen, dit komt omdat bij deze genen ook enkele exons uit het RNA geknipt kunnen worden. Een exon is dus een deel uit een gen dat gebruikt kan worden om een eiwit van te maken, maar het is niet altijd nodig om alle exons van een gen in het RNA te hebben om een bruikbaar eiwit te vormen. Naar mate er meer exons verwijderd worden, wordt het RNA steeds kleiner en het eiwit dat er van gemaakt wordt ook kleiner. Een eiwit waarbij ten opzichte van het RNA dat alle exons bevat, exons zijn verwijderd heet een (alternatieve) splice-variant.

Aminozuren zijn de bouwstenen voor eiwitten, er zijn 20 verschillende aminozuren. Ieder eiwit bestaat uit een aaneenschakeling van aminozuren, de meeste eiwitten bevatten er enkele honderden. De eigenschappen van een eiwit worden bepaald door de volgorde van de aminozuren, die wordt bepaald door de volgorde van de basen in het RNA en die wordt weer bepaald door de volgorde van de basen in het DNA.

14-3-3 eiwitten zijn een familie van eiwitten die erg veel op elkaar lijken (homologen), omdat ze tijdens de evolutie niet veel veranderd zijn. 14-3-3 eiwitten komen in alle tot nu toe onderzochte eukaryotische organismen voor (o.a mensen, dieren, planten, en gist). Er zijn zeven menselijke 14-3-3 isovormen (eiwitten uit dezelfde genfamilie) namelijk 14-3-3 β , γ , ϵ , η , σ , τ en ζ , die tot expressie komen (er wordt een eiwit gemaakt) van verschillende genen. Door te binden aan andere eiwitten reguleren 14-3-3 eiwitten veel processen die belangrijk zijn in de celbiologie zoals; het doorgeven van signalen (signaal transductie), het aanmaken van RNA (transcriptie), apoptose of geprogrammeerde celdood (cellen die overbodig of

beschadigd zijn worden netjes opgeruimd door middel van een soort zelfmoord mechanisme), celcyclus regulatie (de celcyclus is nodig voor het delen van cellen en kan tijdelijk stilgelegd worden) en differentiatie (het ontwikkelen van cellen tot cellen die een gespecialiseerde taak hebben).

De algemene eigenschappen van 14-3-3 eiwitten worden besproken in **hoofdstuk 1 (chapter 1)**. In dit hoofdstuk wordt extra aandacht besteed aan de rol van 14-3-3 eiwitten in keratinocyten (huidcellen) omdat een groot deel van het onderzoek in dit proefschrift gedaan is in, en betrekking heeft op, keratinocyten.

De laatste jaren worden er steeds meer verbanden gevonden tussen 14-3-3 eiwitten en leden van de p53 genfamilie van transcriptiefactoren (eiwitten die transcriptie regelen). De p53 familie bestaat uit drie genen: p53, p63 and p73. Hoewel de p53 familie maar uit drie genen bestaat kunnen er veel meer dan drie verschillende eiwitten van gemaakt worden. Dit komt omdat er van p53, p63 en p73 verschillende splice-varianten zijn. Deze eiwitten spelen een belangrijke rol bij processen in de cellen die ook geregeld worden door 14-3-3 eiwitten.

Een introductie over de p53 familie en de directe verbanden tussen de p53 familie en 14-3-3 eiwitten wordt gegeven in **hoofdstuk 2**. Hier is extra aandacht besteed aan de rol van p53 familieleden in keratinocyten.

Omdat alle 14-3-3 eiwitten op een paar kleine verschillen na precies hetzelfde zijn, wordt er vaak van uit gegaan dat alle 14-3-3 eiwitten op een vergelijkbare manier werken. Desalniettemin zijn er toch aanwijzingen gevonden die er op wijzen dat er ook specifieke taken zijn voor de verschillende 14-3-3 isovormen.

In **hoofdstuk 3** is onderzocht of een van de isovormen, 14-3-3 ζ , specifieke taken heeft bij het ontstaan van kanker door in een keratinocyten cellijn (huidcellen die in een laboratorium gekweekt kunnen worden) 14-3-3 ζ uit te schakelen. Hier is gevonden dat als je 14-3-3 ζ uitschakelt, de cellen veel sneller in apoptose gaan na UV bestraling (er gaan meer cellen dood als je ze met UV licht bestraalt). Ook is

gevonden dat de cellen na uitschakelen van 14-3-3 ζ beter aan elkaar hechten. Bij kankercellen zijn deze twee eigenschappen vaak verloren gegaan. Dit wijst erop dat teveel 14-3-3 ζ ervoor zou kunnen zorgen dat een cel makkelijker een kanker cel kan worden. Er is ook gevonden dat 14-3-3 ζ verhoogd aanwezig is in tumoren, deze vinding ondersteunt het idee dat als 14-3-3 ζ verhoogd in de cel aanwezig is dat de cel makkelijker omgevormd kan worden tot kanker cel.

14-3-3 eiwitten kunnen van het cytoplasma (grotendeels de cel behalve de kern) naar de kern (midden van de cel, hierin bevindt zich het DNA) gaan en vice versa. 14-3-3 eiwitten bevinden zich voornamelijk in het cytoplasma van de cel, maar een klein gedeelte bevindt zich ook in de kern. Verschillen tussen de 14-3-3 isovormen in de snelheid van in en uit de kern gaan zouden kunnen bepalen of 14-3-3 isovormen specifieke biologische functies kunnen hebben. Daarom is in **hoofdstuk 4** in verschillende soorten gekweekte zoogdiercellen onderzocht of er verschillen zijn tussen twee 14-3-3 isovormen, 14-3-3 ζ en 14-3-3 σ , in de hoeveelheid eiwit in de kern en in het cytoplasma en of dat komt omdat de een sneller de kern in- of uitgaat. Hier is gevonden dat er een verschil is in de hoeveelheid 14-3-3 σ in de kern ten opzichte van de hoeveelheid 14-3-3 ζ . Hoewel beide meer voorkomen in het cytoplasma dan in de kern is er naar verhouding meer 14-3-3 ζ in de kern dan 14-3-3 σ . Dat verschil komt doordat 14-3-3 σ sneller de kern uit kan gaan dan 14-3-3 ζ , terwijl ze beiden even snel de kern in gaan.

14-3-3 σ wordt beschouwd als de meest afwijkende vorm van de menselijke 14-3-3 eiwitten. In tegenstelling tot andere isovormen die in bijna alle celtypen tot expressie komen, komt 14-3-3 σ eiwit alleen voor in epitheel cellen (bepaalde groep cel-typen, o.a keratinocyten) en deze isovorm heeft een specifieke rol in de differentiatie van keratinocyten (hoofdstuk 1). In andere celtypen zijn verbanden gevonden tussen p53 familieleden en 14-3-3 eiwitten, in het bijzonder 14-3-3 σ (hoofdstuk 2). In colorectale (darm) kankercellen wordt 14-3-3 σ getransactiveerd (er wordt meer van gemaakt) door p53 na bestraling. Dit is nodig voor een goede arrest (cellen stoppen na bestraling tijdelijk met delen) in de G2 fase van de celcyclus (de celcyclus kent een aantal fasen, G2 is de laatste fase voor de celdeling).

In **hoofdstuk 5** is een mogelijke rol onderzocht voor 14-3-3 σ transactivatie of stabilisatie in door bestraling en p53 veroorzaakte G2 cel-cyclus arrest (een eiwit bestaat maar een korte periode, dan wordt het door de cel afgebroken. Als een bepaald eiwit langer nodig is dan normaal, kan het gestabiliseerd worden, het wordt dan minder snel afgebroken en daardoor blijft er meer van dit eiwit over in de cel). Dit is onderzocht in een p53 gereguleerde keratinocyten cellijn (deze cellijn heeft normaal geen p53 maar p53 kan wel aangezet worden). Hier is gevonden dat p53 nodig is om cellen in een normale G2 arrest te laten komen na bestraling, maar dat dit in deze cellen niet veroorzaakt wordt doordat 14-3-3 σ hoger tot expressie (meer RNA) komt of doordat het eiwit gestabiliseerd wordt. Dit verschil zou kunnen komen omdat 14-3-3 σ bij keratinocyten erg belangrijk is voor differentiatie en omdat daardoor de rol bij cel-cyclus arrest wat minder duidelijk is dan bij andere celtypen.

In **hoofdstuk 6** is onderzocht of een dubbel GFP (groen fluorescerend) eiwit in met p53 getransfecteerde (transfectie is het tijdelijk inbrengen van DNA in cellen dat normaal gesproken niet voorkomt in de cel) cellen blijft zitten nadat de cellen zijn behandeld om het DNA te kleuren. Als p53 en een groen fluorescerend eiwit samen getransfecteerd worden kun je er vanuit gaan dat de groen fluorescerende cellen ook p53 tot expressie brengen, hierdoor is het makkelijker om de functie van p53 te onderzoeken. Het DNA moet gekleurd worden om te kijken in welke fase van de cel-cyclus de cellen zich bevinden en of de cellen in apoptose gaan (deze processen worden gereguleerd door p53). Het is gebleken dat een enkel groen fluorescerend eiwit de cellen uit gaat na de behandeling om het DNA te kleuren. In dit hoofdstuk is gevonden dat een verdubbeling van het GFP eiwit inderdaad toereikend is om een GFP eiwit te maken dat in de cel blijft na DNA kleuring. Dit dubbele groene fluorescerende eiwit kan erg handig zijn bij verder onderzoek naar de precieze functie van p53, p53 familieleden en 14-3-3 eiwitten.

In het laatste hoofdstuk, **hoofdstuk 7**, is een veel gebruikte methode om verschillende p73 vormen (die o.a betrokken zijn bij transactivatie van 14-3-3 σ) aan te tonen bestudeerd. Hier is gevonden dat deze methode wel gebruikt kan worden om te bepalen welke vormen van p73 er aanwezig zijn, maar dat je niet kunt bepalen in welke verhouding deze vormen voorkomen. Hierdoor blijkt het niet mogelijk om naar aanleiding van verschillen in expressie van p73 splice-varianten die gevonden zijn met deze methode uitspraken te doen over de rol van p73 varianten bij ziekten zoals bijvoorbeeld leukemie.

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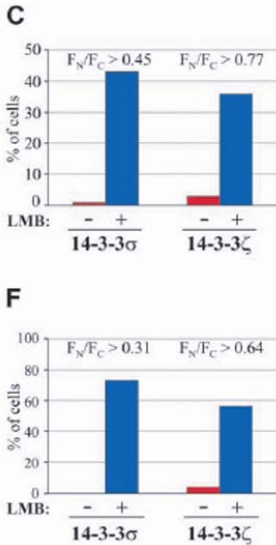
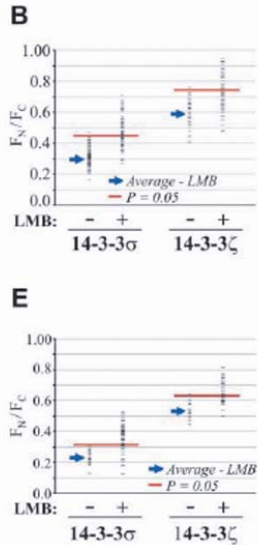
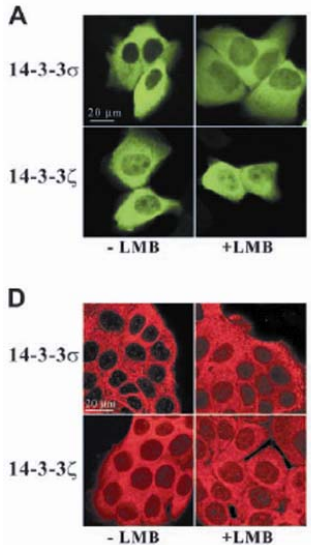
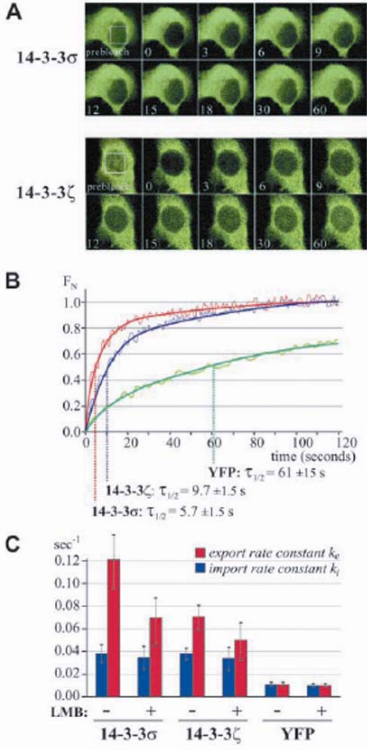
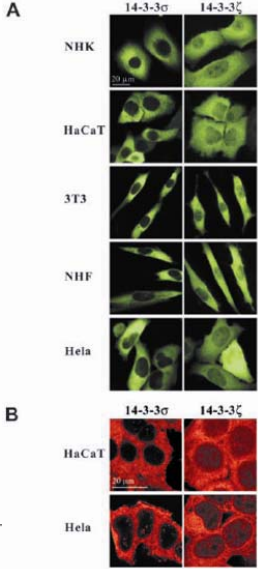
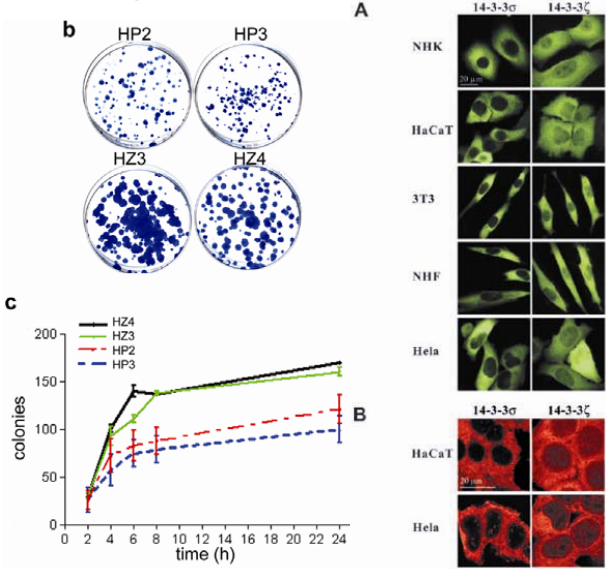
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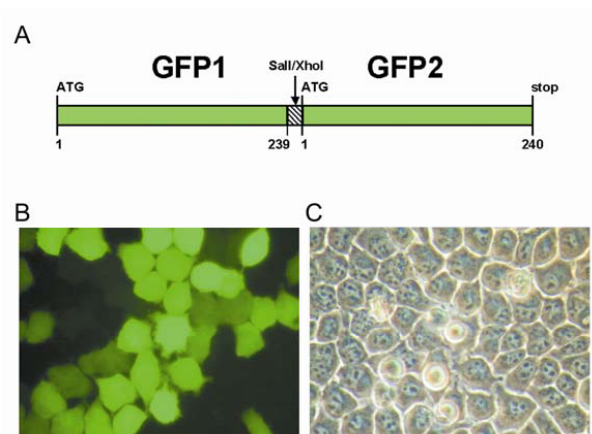
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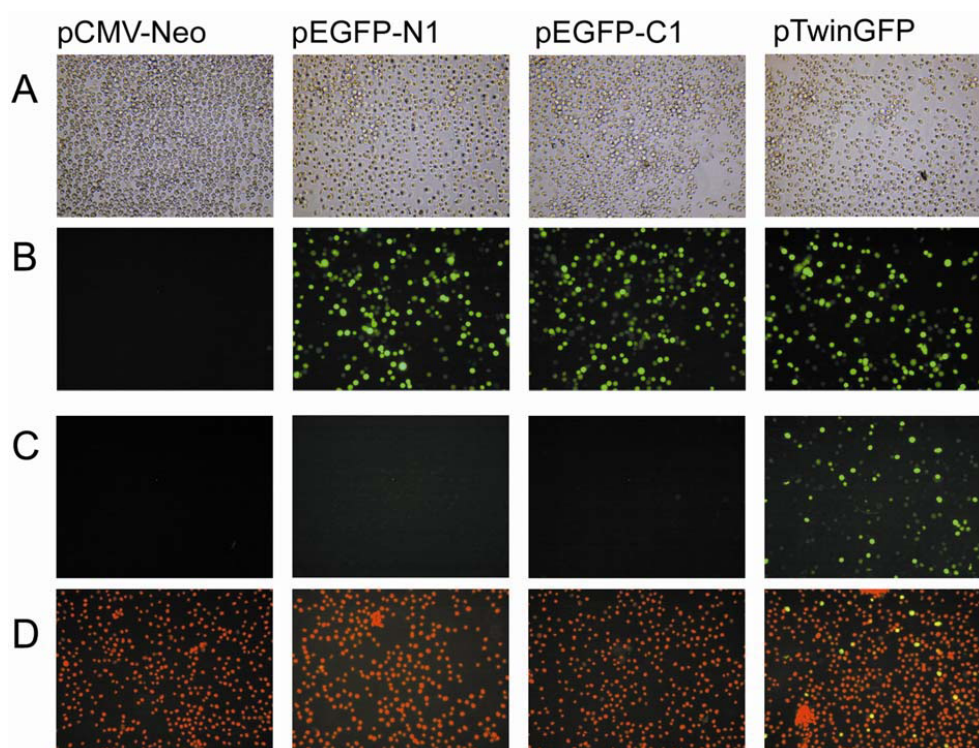
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Color figures





Chapter 6, figure 1



Chapter 6, figure 2

Conclusions and future directions

In recent years significant progress has been made in discovering targets of the 14-3-3 family of proteins, elucidating some of the molecular mechanisms that underlie 14-3-3 function and several important associations between 14-3-3 proteins and members of the p53 family have been revealed. It is evident that both families are key players in major cellular events such as the cell-cycle, apoptosis, differentiation and development and that the two families influence processes regulated by members of the other family. P53 family members regulate expression of genes involved in these events (either positively or negatively) and 14-3-3 proteins bind proteins involved in these processes (including p53 family members), thereby altering their characteristics. However, numerous questions regarding 14-3-3 proteins, p53 family members and the relationships between the two families remain. Such as; why do 14-3-3 proteins and p53 family members express so many different isoforms or splice variants? In which cell-types and under which conditions are these variants expressed exactly? Does each protein exhibit specific biological functions? What is the degree of functional overlap between isoforms or splice-variants? How cell-type specific are the actions carried out by these proteins?

The data presented in this thesis contribute to answer these questions. In chapter 3 isoform-specific characteristics of 14-3-3 ζ have been revealed. A possible role for this protein in carcinogenesis was suggested because downregulation of 14-3-3 ζ increased the propensity for DNA-damage induced apoptosis and increased cell-cell contacts, two characteristics that are often lost in cancer cells. In addition, it is shown that 14-3-3 ζ protein is overexpressed in several tumors, which also indicates that it might be involved in tumorigenesis. In chapter 4, differences between 14-3-3 σ and 14-3-3 ζ in nucleocytoplasmic shuttling have been described. The difference in nucleocytoplasmic shuttling likely forms a basis for specialized biological functions of the 14-3-3 isoforms. In chapter 5, keratinocyte specific p53 regulated G2 cell-cycle arrest and the lack of a contribution for 14-3-3 σ in this cell-type has been observed, whereas 14-3-3 σ induction clearly plays a distinct role in G2 arrest in other cell-types. In chapter 6, an improved method for detecting p53 induced apoptosis by flow

cytometry is portrayed by expressing a duplicated GFP protein. This double GFP protein can be used for future research in the fields of 14-3-3 and p53. In chapter 7, a widely used method to detect and quantify p73 splice-variant expression is critically assessed. It turns out that, using this method it is not possible to accurately quantify p73 splice-variants. This observation might help future research to monitor p73 splice-variant expression more accurately (by using alternative methods) and to prevent inaccurate statements about specific roles for particular p73 splice-variants in diseases such as leukemia.

Although some information about the 14-3-3 and p53 families has become more clear in this thesis, much more research is needed to form a complete picture of how 14-3-3 isoforms and p53 family members collaborate in regulating major cellular processes. Fortunately, many researchers have noticed the scientific importance of 14-3-3 proteins and of the p53 family. For p53, the relevance for cancer biology has been acknowledged for decades. However, recent work has also suggested the involvement of the other p53 family members and the 14-3-3 proteins in human disease, mainly in cancer and neurological disorders and it is likely that other diseases will continue to come forward in the near future. Information concerning expression or activity of various 14-3-3 isoforms and p53 family members is likely to prove useful in the staging and prognostic evaluation, as well as the selection of treatment for certain cancers and possibly other diseases. Therapeutic approaches targeting 14-3-3 proteins are also under consideration. It has been proposed that targeting the 14-3-3 family as a whole might give optimal benefits in therapeutic applications. However, important isoform-specific 14-3-3 biological activities have been discovered, activities that sometimes even counteract the activities of other isoforms. As the knowledge of isoform-specific functions increases, approaches that target the activity of one isoform without interfering with the activity of the others might give straightforward therapeutic benefits. For example, as suggested in chapter 3, because 14-3-3 ζ downregulation reduces two cancer cell specific characteristics, this isoform might be a possible specific target in the future.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 4 Oktober 1976 te Hellevoetsluis. In 1995 haalde hij zijn HAVO diploma aan de Jacob van Liesveldt school, eveneens te Hellevoetsluis. Hierna studeerde hij Biochemie aan het Hoger Laboratorium Onderwijs te Delft aan de Hogeschool Rotterdam en Omstreken, later Hogeschool Rotterdam. Het laatste jaar van de opleiding bestond uit een afstudeerstage die gedaan is aan de Universiteit van Leiden afdeling Moleculaire Genetica die onder verantwoordelijkheid staat van Prof. dr. J. Brouwer. Hier werd onder begeleiding van Dr. J. A Brandsma Nucleotide Excision Repair in de gist *Saccharomyces cerevisiae* bestudeerd. In september 2000 startte hij zijn werk als AIO, eveneens bij de afdeling Moleculaire Genetica, onder begeleiding van Dr. C. Backendorf waar het in dit boekje beschreven promotieonderzoek werd uitgevoerd. Heden is hij aangesteld door Prof. dr. J. A. Langendijk bij de afdeling radiotherapie van het UMCG (Groningen) en voert zijn werkzaamheden als post-doc uit onder begeleiding van Dr. R. P. Coppes bij de afdeling Straling en Stress Celbiologie van Prof. dr. H. H. Kampinga.