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Chaperone Association with Telomere Binding Proteins

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

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List of Abbreviations

17-AAG	17-(Allylamino)-17-demethoxygeldanamycin
17-DMAG	17-NN-Dimethyl-Ethylene Diamine-Geldanamycin
53BP1	p53 binding protein 1
ALT	alternative lengthening of telomeres
APB	ALT associated PML bodies
ATM	ataxia telangiectasia mutated
CDK4	cyclin-dependent kinase 4
ChIP	chromatin immunoprecipitation
CK2	casein kinase 2
DNA-PKcs	DNA-dependent protein kinase
DNTRF2	dominant negative TRF2
DSB	double strand break
DMSO	dimethyl sulfoxide
ERCC1/XPF	excision repair cross-complementing group 1/xeroderma pigmentosum, complementation group F

FISH	fluorescent in situ hybridization
GA	geldanamycin
HIF1	hypoxia-inducible factor 1
HR	homologous recombination
Hsp	heat shock protein
hTERT	human telomerase reverse transcriptase
IP	immunoprecipitation
IR	irradiation
MMP2	matrix metalloproteinase 2
MRE11	meiotic recombination 11
MRN	MRE11-RAD50-NBS1 complex
NBS1	Nijmegen breakage syndrome protein 1
PNA	peptide nucleic acid
PARP	poly(ADP-ribose) polymerase
PIP1	POT1 interacting protein
Plk1	Polo-like kinase 1
PML	promyleocytic leukemia

POT1	protection of telomeres 1
PTOP	POT1 and TIN2 organizing protein
RAD50/51	RAS associated with diabetes GTP-binding protein family 50/51
RAD	Radical
Rap1	human TRF2 interacting telomeric protein
RRL	rabbit reticulocyte lysate
SDS-PAGE	sodium dodecyl (lauryl) sulfate-polyacrylimide gel electrophoresis
SENP	SUMO specific protease
SMC5/6	structural maintenance of chromosomes 5/6
Tank1/2	tankyrase1/2
TIF	telomere induced foci
TIN2	TRF1 interacting factor 2
TINT1	TIN2 interacting protein
TnT	transcription and translation system
TPP1	PTOP/TINT1 protein
TRF	terminal restriction fragment
TRFH	telomere repeat binding factor homology

TRF1	telomere repeat binding factor 1
TRF2	telomere repeat binding factor 2
TRAP	telomerase repeat amplification protocol
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

ABSTRACT

Chaperone Association with Telomere Binding Proteins

By Amy Nicole Depcrynski

A dissertation submitted in partial fulfillment of the requirements for the degree of
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Virginia Commonwealth University, 2009

Director: Shawn E. Holt, PhD, Associate Professor Departments of Pathology, Pharmacology
and Toxicology and Human and Molecular Genetics

The Hsp90 chaperone complex associates with the telomerase enzyme, facilitating the assembly of the ribonucleoprotein complex. While previous data from our laboratory indicate that Hsp90 and p23 remain stably associated with (functionally active) telomerase, more recent experiments suggest that these chaperones associate with telomeres independent of telomerase, presumably through a specific interaction with telomere binding proteins. The current study examines the novel interactions between TRF2, TRF1, TIN2 and TPP1 and molecular chaperones (Hsp90, Hsp70, p23). *In vitro* and in cell experiments have shown an interaction

between TRF1 and TRF2 and the molecular chaperones Hsp90 and Hsp70. Inhibition of Hsp90 using drugs that specifically block ATPase activity results in an increased association of TRF1 and TRF2 with Hsp90 to presumably stabilize the telomere associated proteins to the telomere. A definitive explanation as to the mechanisms underlying the chaperone/telomere associated protein interaction has yet to be determined and further studies examining chaperones' contribution to telomere structure and function are underway. A better understanding of the telomeric proteins and Hsp90 and their roles in nuclear events is important, as both have extremely important functions in the cell.

Our current working hypothesis is that chaperone proteins associate with TRF2, TRF1, TIN2 and TPP1 to facilitate telomeric protein-protein interactions and protein-telomere binding in both cancer and normal cells. The interaction between chaperones and telomere binding proteins may eventually provide a better understanding of telomeric structure and function. Defining the mechanisms of telomeric protein regulation is important in the development of new therapeutic approaches for targeting telomeres to induce dysfunction. Clinical trials are underway employing drugs targeting Hsp90 in cancer cells and given the results here, these Hsp90 compounds likely cause telomere alterations.

Chapter 1

Review of Current Literature

TELOMERE PROTECTION

Telomere dysfunction and the t-loop

Telomeres, the ends of linear eukaryotic chromosomes, play a major role in maintaining chromosomal stability and may provide a target for cancer treatment and/or prevention. Muller and McClintock (1938, 1941) first theorized that telomeres act to distinguish the ends of the chromosomes as natural ends, not damaged DNA, and protect the end of the chromosome. Telomeric DNA is a nucleoprotein complex made up of non-coding DNA which, in humans, ranges in size from 2kb-20kb (Cech, 2009) and consists of tandem repeats of short, double stranded, guanine rich sequences of TTAGGG, a single stranded 3' overhang, and associated proteins. These components allow the telomere to form the t-loop to differentiate the ends from broken DNA, which is crucial in preventing the activation of double-strand break repair mechanisms. Two telomeric proteins, TRF1 and TRF2, bind directly to the telomeric DNA and complex with other proteins including TIN2, Tankyrase1/2, TPP1, Rap1, POT1 and the Mre11 complex (Figure 1, top panel). Together they function to organize the linear chromosomal ends into the protected t-loop structures (Figure 1, bottom panel) (Griffith, *et al.*, 1999). Investigators have shown these proteins also regulate length and help to prevent genetic instability that has been associated with short telomeres.

The structure of the t-loop is formed by the end of the telomere folding back on itself. This circular segment is made up of duplex telomeric DNA bound by TRF1. The 3' overhang of single stranded telomeric DNA invades the duplex DNA so that the TTAGGG repeat strand is

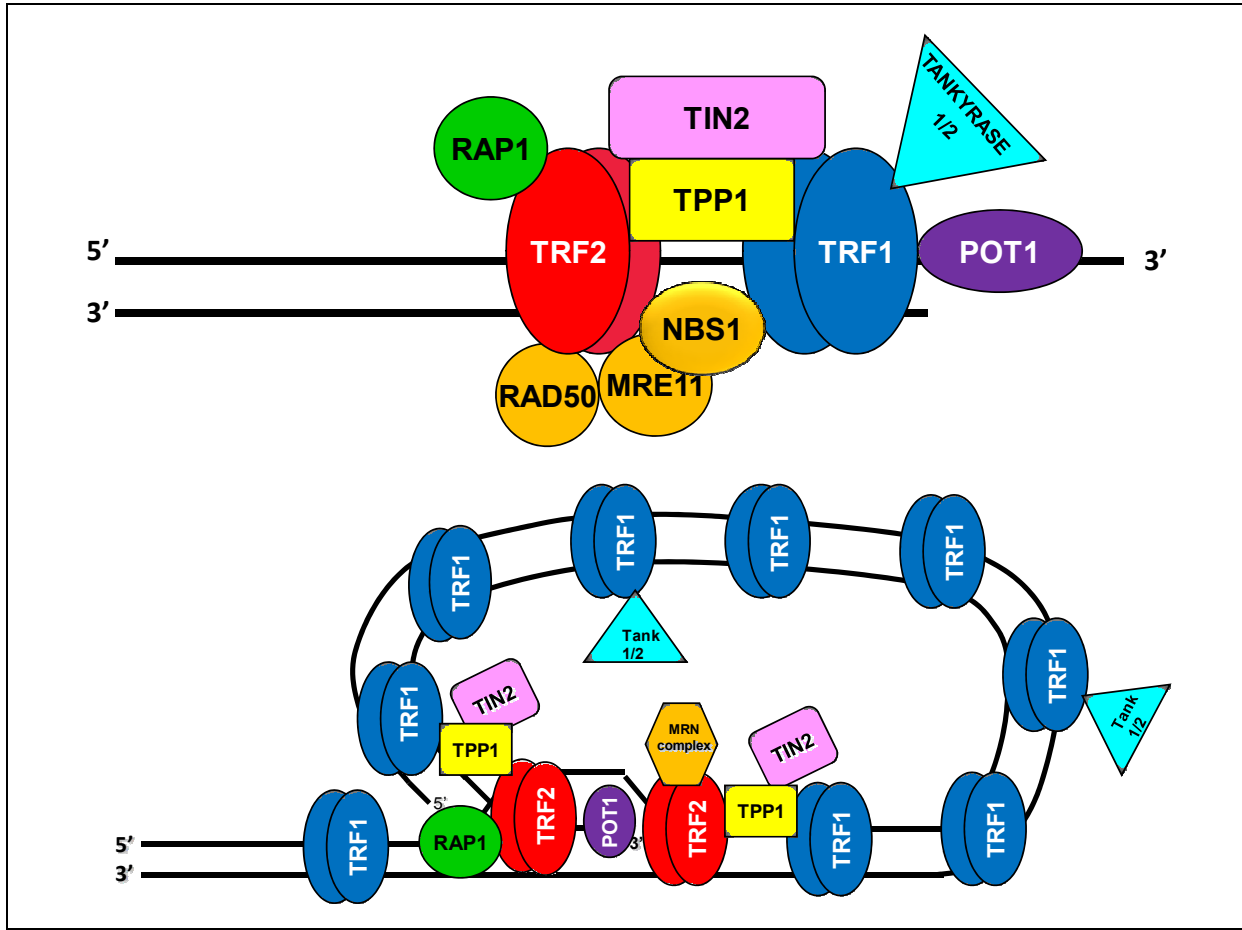


Figure 1. The telomere structure and associated proteins. Top panel shows telomeric binding proteins and their known interactions with associated proteins. TRF1 and TRF2 bind the telomeric DNA, while many of the associated proteins interact with TRF1 and/or TRF2 without binding the DNA directly. Bottom panel displays the hypothesized t-loop structure, indicating that TRF2 aids in the invasion of the 3' overhang into the duplex DNA while TRF1 coats the telomeric DNA. Adapted from de Lange, 2005.

displaced at the loop-tail junction. TRF2 appears to promote this loop formation by facilitating the invasion of the 3' end, while TRF1 helps to coil the duplex DNA on itself (Figure 1, bottom panel). Tankyrase, a telomeric poly(ADP)ribose polymerase, inhibits the binding of TRF1 to the telomere and may therefore also function in the regulation of the t-loop (Smith, *et al.*, 1999). Sequestering the telomeric ends into these t-loop structures prevents cellular exonuclease activities and telomerase from acting on the DNA (Griffith, *et al.*, 1999). Shortened telomeres fail to form t-loops, contributing greatly to genomic instability. As evidenced through the use of a dominant negative TRF2 (DNTRF2) mutant, open t-loops induce the activation of DNA checkpoints, including signaling through ATM and p53 (Karlseder, *et al.*, 1999).

With each cell division telomeric DNA is progressively lost due to the end replication problem, in which normal lagging strand replication fails to completely copy chromosome ends, leaving a gap between the final RNA priming event and the terminus. As this shortening continues over many cell divisions, the telomeres reach a critical length where they elicit a cellular senescence response in normal human cells (Levy, *et al.*, 1992) Critical shortening results in dysfunctional telomeres in cells that have bypassed the senescence checkpoint and cause chromosomal instability, resulting in end-to-end fusions, dicentrics, breakage of DNA, missegregation, and chromosomal rearrangements (Counter, 1996; McClintock, 1941). Telomere shortening often results in extremely high levels of genetic instability, which can eventually contribute to tumorigenesis. Cancer cells activate telomerase to aid in lengthening telomeres, resulting in cellular immortalization. Short telomeres have been found in many carcinomas, including invasive breast cancers (Odagiri, *et al.*, 1999; Griffith, *et al.*, 1999), basal cell carcinoma (Han, *et al.*, 2009), hepatocarcinomas, lung carcinomas (Oh, *et al.*, 2005; Matsutani, *et al.*, 2001), prostate cancer (Meeker, *et al.*, 2002), bladder cancer (Wu, *et al.*, 2003), esophageal

cancer (Shammas, *et al.*, 2004), and gastric cancer (Liu, *et al.*, 2009), suggesting that telomerase can maintain short but functional telomeres in tumor cells. This evidence also suggests that telomeric proteins and stable t-loops play a major role in protection against cancers.

Telomeric Proteins

Described as the Shelterin protein complex, the telomere binding proteins TRF1, TRF2, POT1 (Protection of Telomeres 1) and associated proteins TIN2, Rap1, and TPP1, are defined as proteins abundant at the telomere but not elsewhere, present throughout the cell cycle, and functioning only at the telomere, thereby having high specificity to the telomeric repeats (de Lange, 2005). The six components of the Shelterin complex fractionate together, with TIN2 tethering TPP1 to TRF1 and TRF2 thereby connecting TRF1 to TRF2 for stability. This, in turn, directly binds Rap1 (Liu, *et al.*, 2004; Ye, *et al.*, 2004). POT1 is the only Shelterin protein to bind the 3' single strand overhang of the telomere and also associates with TRF1 (Kelleher, *et al.*, 2005). Proteins associated with the telomere that do not fall into the criteria of Shelterin components include: Mre11/Rad50/Nbs1, ERCC1/XPF, the WRN helicase, the BLM helicases, DNA-PKcs, PARP2, Tankyrase 1 and 2, PINX1 and RAD51D, many of which are classified as DNA damage proteins (Table 1).

Telomere repeat binding factors 1 and 2 (TRF1 and TRF2) are paralogs that bind directly to the double strand region of the telomere (Chong, *et al.*, 1995; Bilaud, *et al.*, 1996; Broccoli, *et al.*, 1997; Bilaud, *et al.*, 1997) and play a major role in regulating the length of the telomere (van Steensel and de Lange, 1997; Smorgorzewska, *et al.*, 2000). Their domain architecture is similar in that they share a hinge region, known as the TRF homology (TRFH) domain, which contains amino acids for homodimerization or homomultimerization. This hinge region brings together

Table 1. Telomere-associated proteins (reviewed in Karleseder, *et al.*, 2003).

Protein	Function at Telomere	kDa	pI
TRF1	Sequester chromosome ends in t-loop, coat telomere	60	6.5
TRF2	Major protective factor, 3' overhang invasion to t-loop	65/69	9.2/9.22
TIN2	Capping, length control	40	9.21
hPOT1	Regulates telomere elongation via telomerase, binds TRF1	72	6.82
PTOP/PIP1	Binds TRF1 and TRF2, blocks telomerase access to telomere	58	4.38
hRAP1	Interacts with TRF2, t-loop/elongation regulation	50	7.02
MRE11	Regulation of t-loop, interacts with TRF2	81	5.82
NBS1	Regulation of t-loop, interacts with TRF1 and TRF2	95	6.91
RAD50	Regulation of t-loop, interacts with TRF2	180	6.89
Tank 1/2	Inhibits TRF1 binding to telomere, regulation of t-loop/telomerase	100/ 116	7.05/ 7.2
Ku70/80	Regulates capping	70/80	6.7/5.8
XRCC1	Resistance to DNA damaging agents	70	8.83
PARP1	DNA base excision repair and single strand break repair	28	9.88
DNAPKcs	Regulates capping	460	7.8
RAD51D	Length maintenance and regulation	37	6.23
RAD54	Telomere length maintenance	85	8.49
Ku86	Regulates capping and length	86	12.37

the Myb helix turn helix DNA binding domain of both proteins, allowing for sequence specific binding on the telomere (Figure 2). Mutational studies in fission yeast of the telomeric protein Taz1 (an ortholog to TRF1 and TRF2) further implicates the importance of the TRFH domain in telomeric localization, DNA binding and dimerization (Fairall, *et al.*, 2001), as well as association with other telomeric proteins. TRF1 and TRF2 do not heterodimerize and interact only through proteins contained in the Shelterin protein complex (Karlseder, 2003). In fact, the TRFH domain in both TRF1 and TRF2 has a specific recognition motif for different proteins, implicating the consensus sequence YYHKYRLSPL as a means for recruitment of telomere associated proteins. TRF2's TRFH domain recognizes the motif [Y/F]XL, and the TRF2 interacting proteins TIN2 and Apollo, as well as two previously identified proteins, PNUTS and MCPH1, bind this sequence (Kim, *et al.*, 2009).

As mentioned, TRF1 and TRF2 form complexes at the telomeric DNA through protein-protein interactions with telomere associated proteins and other DNA damage proteins. TRF1 binds TIN2 via a domain in the TRF1 homodimerization region (Kim, *et al.*, 1999). TIN2 also exists in a complex with TRF1 and POT1 (Loayza and de Lange 2003). These interactions help to mediate telomere length control activity by TRF1 through modulation of the telomere structure. POT1 is a single stranded DNA binding protein that controls telomerase mediated telomere elongation (Loayza and de Lange, 2003). Another protein, TPP1, known by different names including PTP1, PIP1, and TINT1, recruits POT1 to the TRF1 complex and physically connects TIN2 and POT1 (reviewed by Colgin and Reddel, 2004). TRF1 is also regulated by the Tankyrases (1 and 2) (Smith, *et al.*, 1998; Smith and de Lange, 2000) which inhibit TRF1's protective function at the telomere. Tankyrase1 and 2 promote telomere elongation with long term overexpression and mediate the ADP-ribosylation of TRF1 to open the telomeric complex,

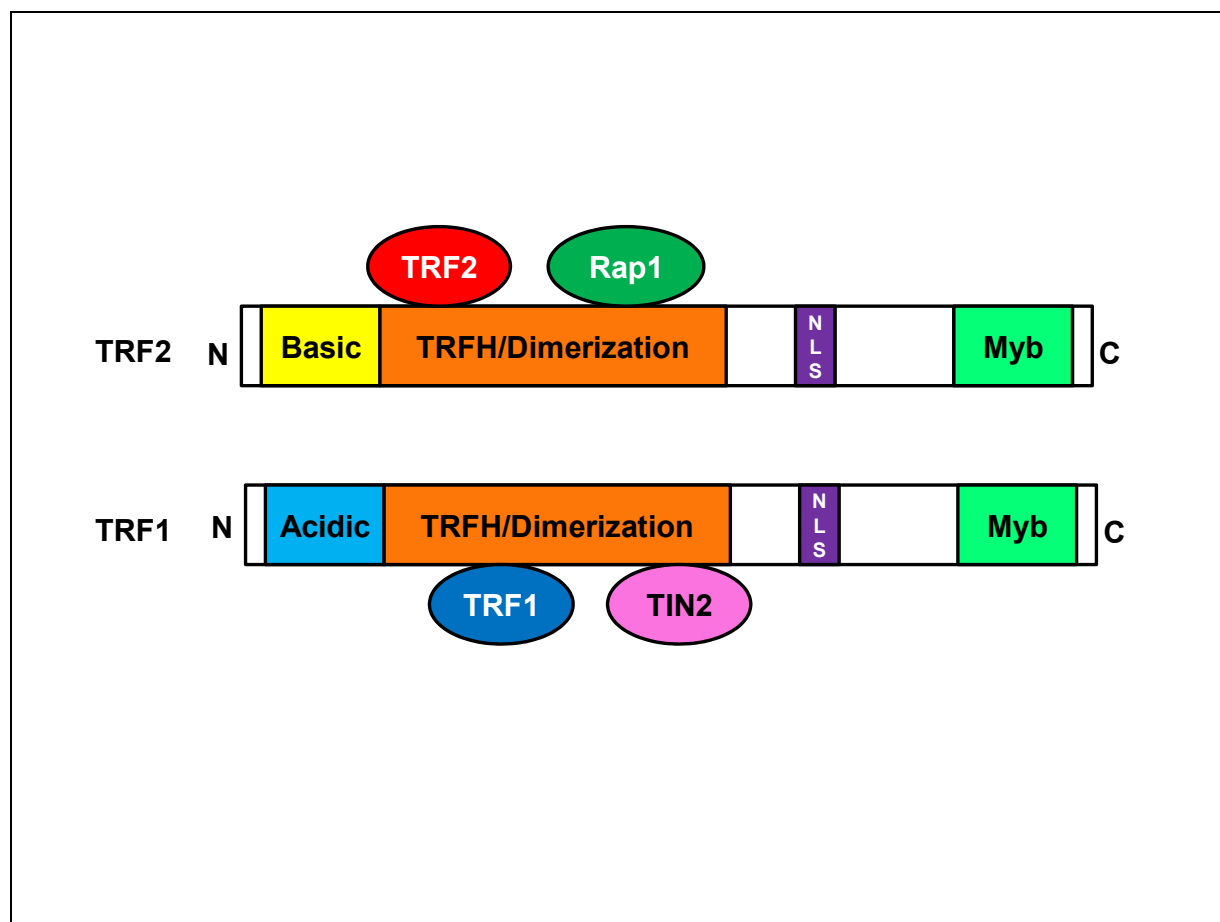


Figure 2. The structure of TRF1 and TRF2. Both TRF1 and TRF2 possess a TRFH/dimerization domain, which contains recognition sequences for interacting proteins and homodimerization, a Myb DNA binding domain that binds the telomeric DNA and nuclear localization sequences. TRF1 differs from TRF2 at the N-terminal, with TRF1 having an acidic N-terminal and TRF2 having a basic N-terminal. Adapted from: de Lange, 2005.

which allows for access to the telomere by telomerase, resulting in elongation (Smith and de Lange, 2000).

TRF2 interacts with many of the same proteins as TRF1, which thereby contribute to the association of TRF1 and TRF2. TIN2 binds TRF2 and mediates its end-capping function, interacts with both TRF1 and TRF2, and forms complexes with TRF1 and other interacting proteins (such as TPP1). Mutants of TIN2 result in a DNA damage response, indicating that the TIN2 complexes are important for both telomere capping and telomere length control (Kim, *et al.*, 2004). TPP1 binds TRF2 as well as TRF1, acting as a secondary barrier against telomerase access to the telomere (reviewed by Colgin and Reddel, 2004). The Mre11 complex, consisting of a trimeric protein made up of Mre11/Rad50/Nbs1, normally helps maintain genomic integrity and processes double stranded breaks (DSBs) (Haber, 1998) and stably interacts with TRF2 (Zhu, *et al.*, 2000). Nbs1 interacts only with TRF2 at the telomeres in S phase, while the rest of the complex interacts throughout the cell cycle and also with TRF1 (Wu, *et al.*, 2000). Possible roles for the complex and its interaction with TRF1 and TRF2 include providing sequence for the generation of 3' overhangs at the telomere, contributing to the formation and maintenance of t-loops, and/or contributing to the controlled resolution of the t-loops (de Lange and Petrini, 2000).

Other proteins associated with the DNA damage response proposed to be associated with TRF1 and TRF2 include the DNA-PK (DNA-dependent protein kinase) complex, made up of Ku70, Ku80, and the catalytic subunit DNA-PKcs (Bailey, *et al.*, 1999; d'Adda di Fagagna, *et al.*, 2001; Goytisolo, *et al.*, 2001; Hsu, *et al.*, 2000; Samper, *et al.*, 2000). DNA-PKcs plays a role in capping the telomere but not in regulation of length of the telomere (Gilley, *et al.*, 2001). Ku80 regulates telomere length and capping; its absence showed a loss of telomeric sequence and increased telomere fusions (Espejel, *et al.*, 2002; Jaco, *et al.*, 2004; Samper, *et al.*, 2000).

The PARP1 DNA repair protein, a poly(ADP-ribose) polymerase whose normal function is in DNA base excision repair and single strand break repair (D'Amours, *et al.*, 1999), also has been found to act at the telomere (d'Adda di Fagagna, *et al.*, 1999). RAD51 paralogs (RAD51B, RAD51C, RAD51D, and XRCC2 and XRCC3) are required in cells for normal levels of genetic recombination and resistance to DNA damaging agents. RAD51D is involved in maintaining and regulating telomere length, and inactivation of RAD51D results in cell death due to telomere uncapping (Tasounas, *et al.*, 2004). RAD54, part of the homologous recombination (HR) machinery also plays a role in telomere length maintenance (Jaco, *et al.*, 2003), although the exact mechanism is currently unknown.

TRF2

The telomeric multiprotein complex organizes the linear chromosome end into a protected t-loop structure, which contributes to telomere length regulation and end protection (Griffith, *et al.*, 1999). One protein in particular, TRF2, aids in protection of telomeres from end-end fusions by directing the invasion of the 3' single strand overhang into duplex telomeric DNA, creating the t-loop (Griffith, *et al.*, 1999; Stansel, *et al.*, 2001). As noted previously, when normal somatic cells divide, telomeres continuously shorten until they reach a critical length, providing less substrate for telomere binding proteins, most notably TRF2, to maintain the t-loop structure and mask the telomere from DNA damage response machinery (Karlseider, *et al.*, 2002). When telomeres are exposed due to critical shortening and the loss of the t-loop, they are recognized as damaged DNA. The resulting telomere dysfunction is associated with characteristic cytogenetic abnormalities: end-end fusions, anaphase bridges, radials, dicentric chromosomes, gene

amplification, and overall genomic instability, eventually leading to immortalization (Karlseder, *et al.*, 2002).

Overexpression of the dominant negative form of TRF2 (TRF2 Δ B Δ M) (Figure 3) demonstrates how important TRF2 functions as a protective factor at the chromosome ends (van Steensel, *et al.*, 1998). Removal of TRF2 from the telomere results in loss of the G strand overhang, which leads to ~15% of telomere ends fusing together, resulting in DNA bridges, fused chromosomes and overall genomic instability in the form of translocations, nondisjunction and aneuploidy (van Steensel, *et al.*, 1998; Smogorzewska, *et al.*, 2002). Absence of TRF2 also leads to both apoptosis and senescence, as well as a DNA damage response involving activation of p53 and expression of p21^{cip1/waf1}, Bax and ATM kinase, so that the cell recognizes the lack of TRF2 as deprotected telomeres or damaged DNA (van Steensel, *et al.*, 1998; Karlseder, *et al.*, 1999). The resulting dysfunctional telomeres are recognized and bound by 53BP1, γ H2AX, RAD17, ATM, and MRE11. These DNA damage protein complexes are referred to as telomere dysfunction induced foci (TIFs). The induction of this DNA damage response is a hallmark of telomere dysfunction and characteristic of inhibition of TRF2 (Takai, *et al.*, 2003).

TRF2 levels are found to be at least two fold higher in breast tumor cell lines, indicating that elevated TRF2 levels are a frequent occurrence in breast cancer (Nijjar, *et al.*, 2005). Overexpression of TRF2 increases the incidence of skin cancer in a transgenic mouse model and also induces premature aging through accelerated shortening of telomeres (Munoz, *et al.*, 2005). Whether this is due to elevated telomerase activation or a decrease in the DNA damage response at short telomeres has yet to be resolved. There is evidence for TRF2 upregulation in a number of other cancers including basal cell carcinomas (Munoz, *et al.*, 2005), breast tumors, hepatocarcinomas, and lung carcinomas (Oh, *et al.*, 2005; Matsutani, *et al.*, 2001). Additionally,

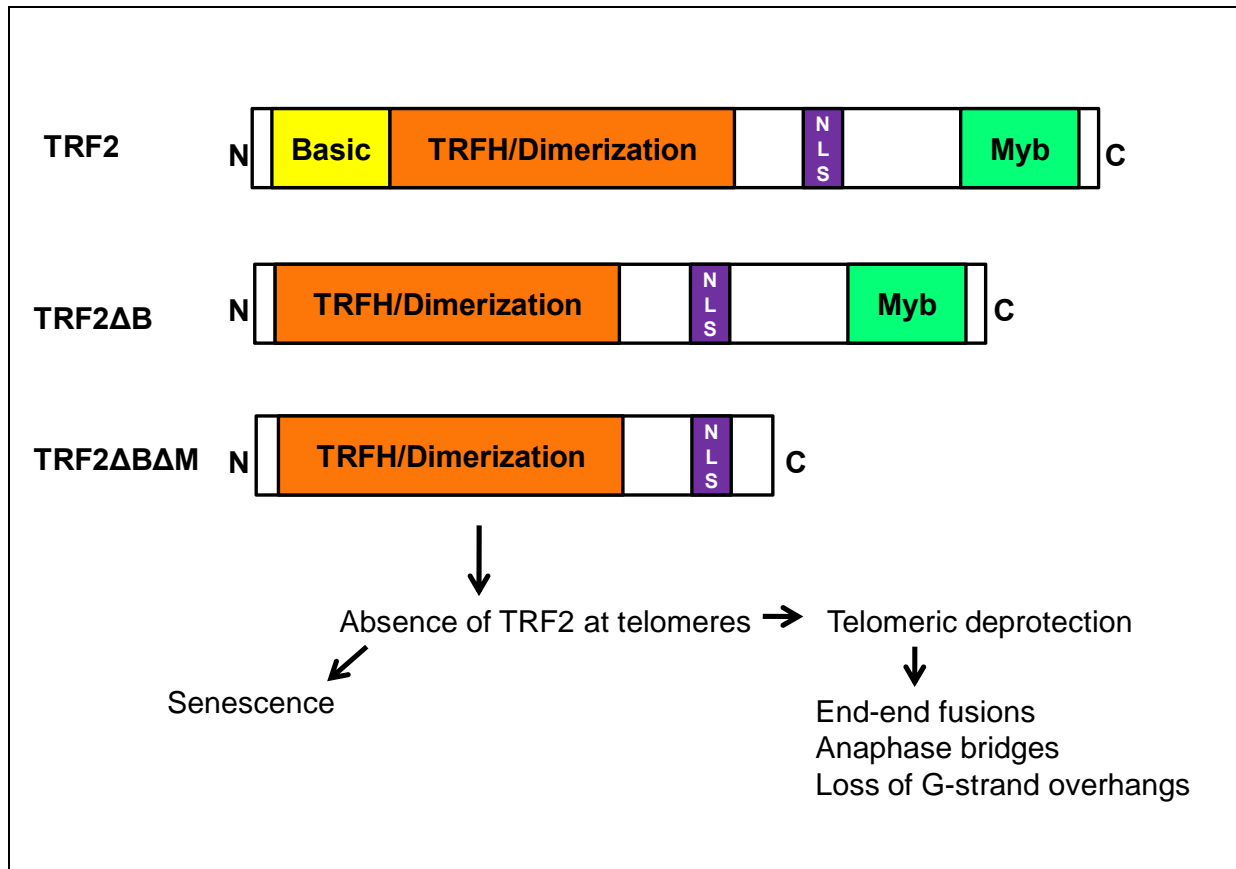


Figure 3. The structure of dominant-negative TRF2. TRF2ΔB lacks the N-terminal basic domain, while the TRF2ΔBΔM lacks both the N-terminal and the C-terminal Myb domain, thus preventing telomeric binding. The resulting absence of TRF2 at the telomere leads to telomere dysfunction and cellular senescence. Adapted from: van Steensel, *et al.*, 1998.

TRF2 has been found at sites of DNA double strand breaks outside of the telomere (Bradshaw, *et al.*, 2005), suggesting that TRF2 plays an important role, not just at the telomere, but at other sites of DNA damage. It may serve as an early indicator of genomic instability that could contribute to tumorigenesis. Elevated levels of TRF2 in various cancer cell lines also indicate a role as a marker for cancer and/or as a target for treatment.

TRF2 has recently been identified as having a role in chromatin organization at the telomere (Figure 4). Telomeres contain nucleosomes that are more compact with shorter weaker spacing than other nucleosomes, similar to pericentric heterochromatin (Tommerup, *et al.*, 1994; Fajkus, *et al.*, 1995). TRF2 can generate positive supercoiling with an ability to condense DNA, requiring both the TRFH domain and the N-terminal DNA binding domain of TRF2. Electron scanning microscopy shows DNA as more condensed around TRF2 complexes, suggesting that TRF2 wraps the DNA around itself. In this nucleosome-type model, TRF2 induces the untwisting of nearby DNA, allowing for strand invasion through positive supercoiling, similar to what likely happens to form the t-loop (Amiard, *et al.*, 2007). In a more recent study, transgenic mice with overexpressed TRF2 showed a decrease in histones H3 and H4, resulting in disrupted nucleosomal spacing specific to telomeric chromatin, suggesting that TRF2 may have an additional impact on nucleosomal organization at the telomere (Benetti, *et al.*, 2008). Previous studies implicated telomere chromatin structure in the negative regulation of telomere length and protection against genomic instability, further indicating a distinct role for TRF2 in telomeric protection (Blasco, 2007; Baker, *et al.*, 2009).

The literature suggests a major role for TRF2 in the protection of the telomere. Although much has been studied as to the effects of loss of TRF2 (through the DNTRF2) and its protein-protein interactions, the mechanisms surrounding how TRF2 protects the telomere have not been

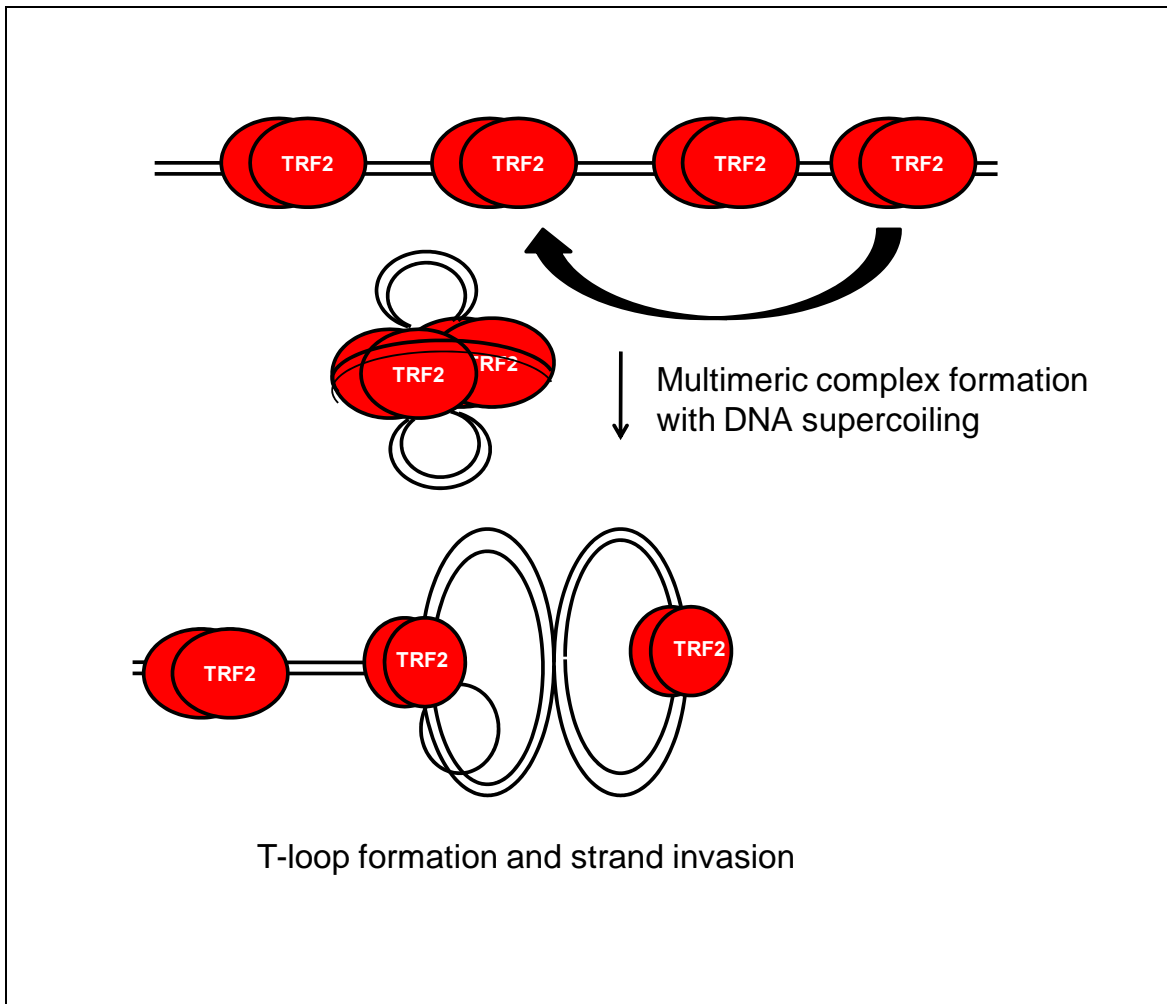


Figure 4. TRF2's role in chromatin remodeling. TRF2 binds the telomeric DNA, forming multimeric complexes that the telomeric DNA wraps around. This leads to positive supercoiling and looping of neighboring DNA, possibly inducing the invasion of single-stranded DNA into the duplex DNA, creating the t-loop structure. Adapted from: Amiard, *et al.*, 2007.

examined as closely. As mentioned above, there is a suggested role in chromatin modeling and t-loop formation. Further exploration is warranted. It is possible that dimerization of TRF2 impacts its ability to bind the telomere or there may be other proteins involved in TRF2's ability to stably associate with both the telomere and other Shelterin proteins. Elucidating these mechanisms may provide a better understanding of TRF2's role in protection of the telomere.

POST-TRANSLATIONAL MODIFICATION OF TELOMERIC PROTEINS

Post-translational modification of telomeric proteins has not been widely researched. However, a number of protein kinases associate at the telomere to aid telomere maintenance. These include ATM and DNA-PKcs (d'Adda di Fagagna, *et al.*, 2004). The following focuses on the telomere binding proteins TRF2 and TRF1 and their post-translational modifications, including phosphorylation, ubiquitination, and SUMOylation.

Phosphorylation

The addition of phosphate groups to a protein normally occurs on a serine, threonine or tyrosine and acts as a regulatory mechanism, often activating a protein that is in turn deactivated by dephosphorylation (as is the case with p53). Phosphorylation can also play a role in protein-protein interactions and in protein degradation. Although few groups have studied the phosphorylation of TRF2, it has been determined that TRF2 is phosphorylated *in vitro* by the protein kinase Aurora C, a member of the Aurora protein family of serine/threonine kinases (Spengler, 2007). This phosphorylation has a role in telomere homeostasis in germ cells, where

Aurora C has previously been identified to function, and in chromatin remodeling at the telomere, as Aurora B has been implicated. However, further studies are required to show a definitive regulation (Spengler, 2007).

As TRF2 is implicated in binding to sites of DNA damage (Bradshaw, *et al.*, 2005), it is highly likely that TRF2 is phosphorylated in response to damage repair. Though a transient association, phosphorylated TRF2 was detected at sites of DNA damage after irradiation (IR), wherein the phosphorylated TRF2 comes off the telomere, associates with ATM and localizes to DSB sites elsewhere on the chromosome. This occurs rapidly, 30 minutes after IR and disappears by 8 hours. Cells that use the ALT pathway or undergo some sort of telomeric crisis also have phosphorylated TRF2 localized to the telomeres. The suggestion that TRF2 can localize to both telomeric damage sites and genome wide DNA damage implicates TRF2 in a global role of DNA damage protection (Tanaka, *et al.*, 2005). Interestingly, the modification by ATM phosphorylation has also been shown to be associated with TRF1 (Kishi, *et al.*, 2001).

TRF1 is a target of phosphorylation for Polo-like kinase 1 (Plk1) through a priming event mediated by the Cdk1 protein kinase (Wu, *et al.*, 2008). Phosphorylation by Plk1 seems to increase TRF1's ability to bind to telomeric DNA *in vitro* as the Plk1 targeting site is within TRF1's DNA binding domain. Chromatin immunoprecipitation (ChIP) experiments using either Plk1 depletion or mutation of the Plk1 targeting site strongly reduced TRF1's ability to bind to the telomere (Wu, *et al.*, 2008). Casein kinase 2 (CK2) mediates phosphorylation of TRF1 and affects telomeric binding *in vitro* as well as *in vivo*. Chemical inhibition of CK2 reduced TRF1's ability to bind to the telomere and TRF1 was subsequently ubiquitinated and degraded, suggesting a role for phosphorylation in regulating normal TRF1 turnover (Kim, *et al.*, 2008). The Akt/protein kinase B complex interacts directly with TRF1 and mediates telomere

shortening when overexpressed in an HTC immortalized liver carcinoma cell line, possibly by upregulating TRF1 levels. Akt's role in genomic instability suggests that this function at the telomere may be a factor in its effects on tumorigenesis (Chen, *et al.*, 2009).

Ubiquitination

Ubiquitination is a form of post-translational modification that involves the attachment of one or more ubiquitin monomer(s) to a protein, usually as a way to label proteins for degradation via the proteasome. Ubiquitination is an ATP-dependent reaction wherein Ubiquitin is activated by the E1 activating enzyme, producing an ubiquitin-adenylate intermediate. Ubiquitin is transferred to the cysteine of the E2 conjugating enzyme after which E3 ubiquitin ligases recognize the substrate and facilitate ubiquitin binding to its target protein (Figure 5) (reviewed in: Hershko and Ciechanover, 1998).

As with phosphorylation of telomeric proteins, studies regarding ubiquitination of TRF1 or TRF2 are rare. Because these two proteins play a large role in regulating telomere length homeostasis, it is likely that these protein levels must be maintained to ensure proper protection against telomerase, particularly regarding TRF1's role in inhibiting telomerase access to the telomere. Two ubiquitin ligase proteins, RLIM and Fbx4, have been implicated in regulating TRF1 turnover by targeting it for proteasome-mediated degradation, thereby regulating the protein levels of TRF1 in the cell. Overexpression of both RLIM and Fbx4 reduced the half life of TRF1, with Fbx4 targeting TRF1 for ubiquitination by acting as an adaptor of the Cul1-based ubiquitin ligase complex (Lee, *et al.*, 2006). The two ligases appear to act independently of one another and bind to different regions of TRF1 but may cooperate to degrade TRF1 when expressed at high levels (Her and Chung, 2009; Lee, *et al.*, 2006).

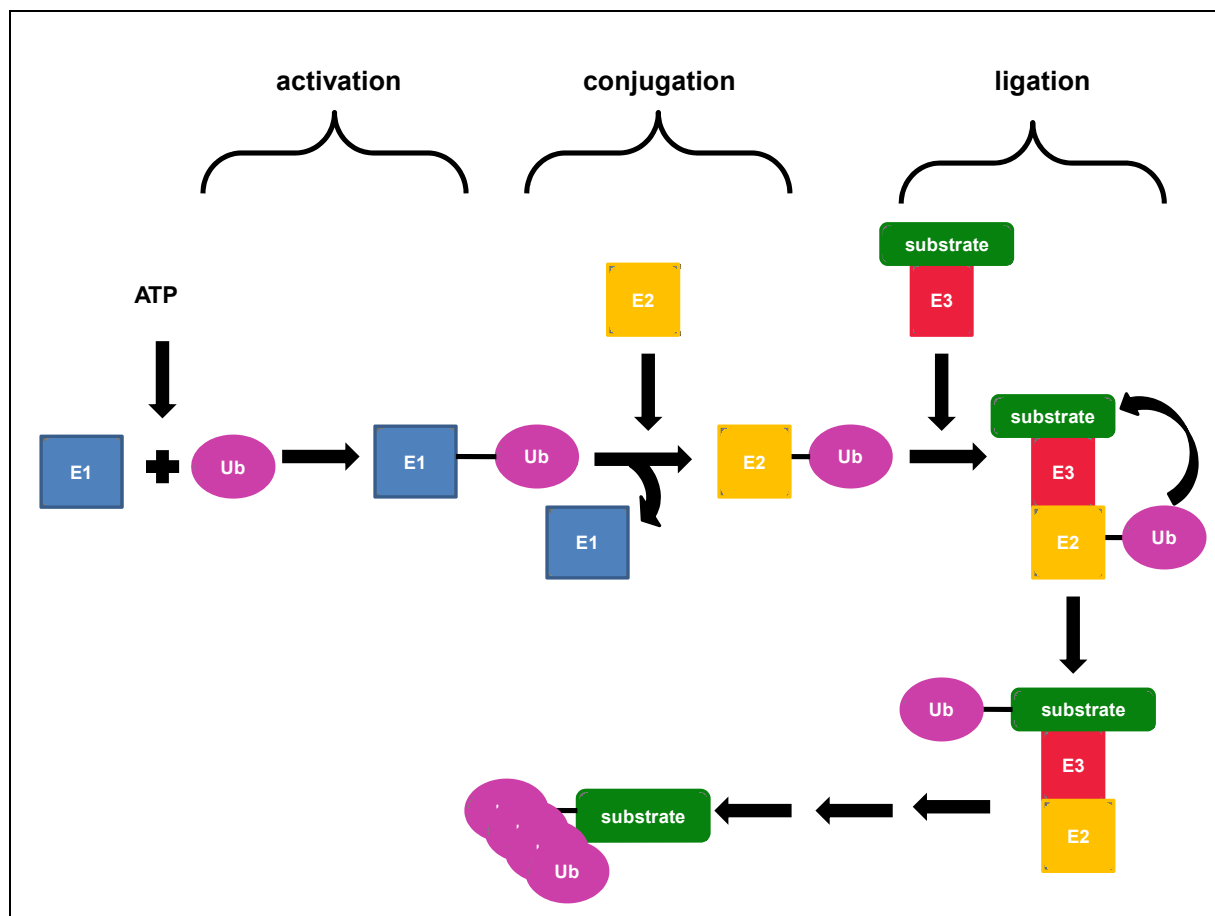


Figure 5. The Ubiquitination Pathway. The pathway begins with the E1 activating-enzyme, which in an ATP dependent reaction activates and binds the glycine residue of ubiquitin and is then transferred to the cysteine of the E2 conjugating enzyme. In the final step, catalyzed by the E3 ligating enzyme, ubiquitin is linked to an ϵ -amino group on the substrate protein's lysine residue. A polyubiquitin chain is then formed, where the C-terminus of the ubiquitin subunit is linked to a lysine residue on the previous ubiquitin. Adapted from: Hershko and Ciechanover, 1998.

TRF1 binds to Tankyrase1, which is a poly(ADP-ribose) polymerase (PARP) that is associated with the telomere. When TRF1 is ADP-ribosylated by Tankyrase1, it is unable to bind to the telomere, thus allowing telomerase access and inducing telomere elongation (Smith, *et al.*, 1998; Smith and de Lange, 2000). Tankyrase1 induces proteasome-mediated degradation of TRF1 both *in vitro* and *in vivo*, where the subsequent release of TRF1 from the telomeres after ADP-ribosylation signals ubiquitination and degradation of TRF1. However, PARP activity is not required for ubiquitination to occur, as this is an independent event that takes place after ADP-ribosylation *in vitro* (Chang, *et al.*, 2003). It appears, in this case, that the unbound TRF1 is being targeted for degradation while being protected from degradation when bound to the telomere.

Implications regarding bound TRF1 interacting with the E3 ligases mentioned above suggest that degradation of only unbound TRF1 is not always the case, and maintaining proper TRF1 levels also may be an important trigger for ubiquitination. A literature search revealed no studies regarding TRF2's ubiquitination, but TRF2 levels at the telomere seem not to be as tightly regulated as TRF1.

Sumoylation

SUMOylation is a form of post-translation modification with a pathway similar to ubiquitin, though its roles in the cell are numerous compared to ubiquitin, including protein-protein interaction, DNA binding, localization, and direction towards degradation. SUMOylation, the conjugation of SUMO to its target proteins, involves binding of the SUMO protein to a consensus sequence containing a ψ KxE motif, where ψ is a hydrophobic amino acid and x is any amino acid. The pathway involves enzymes specific to SUMO, but it is similar to

the ubiquitin pathway. SUMO is synthesized by SUMO specific proteases (SENPs) and is conjugated to target proteins in an ATP dependent manner by the SUMO E1 activating enzyme (AOS1/UBA2) and the E2 conjugating enzyme (Ubc9) (Figure 6) (Wilson and Rangasamy, 2001). E3 ligases also are involved, but seem only to play a role in specificity of target proteins and are not necessary for *in vitro* SUMOylation. SUMO and ubiquitin's roles in the regulation of TRF2 may provide clues into TRF2's actions at the telomere in normal and cancer cells.

Promyelocytic leukemia (PML) bodies of ALT cells (which lengthen the telomere through homologous recombination not telomerase) are also known as ALT-associated PML bodies (APBs) and contain telomeric chromatin, Shelterin proteins, and DNA repair proteins. The results of several studies suggest the SMC5/6 (structural maintenance of chromosomes 5/6) holocomplex has a role in SUMOylation at the telomere, particularly TRF2 SUMOylation in ALT cells. The SMC5/6 complex contains a SUMO ligase called MMS1, which is also found in the APBs (reviewed in Reddel, 2007). MMS1 stimulates SUMOylation of TRF1, TRF2, TIN2 and Rap1 whereas inhibition of TRF1 or TRF2 SUMOylation prevents APB formation altogether. Inhibition of MMS1 or SMC5/6 causes telomere shortening, an increase in end-end fusions and senescence in ALT cells, which inhibits the homologous recombination of the telomere (Potts and Yu, 2007). The SUMOylation of Shelterin suggests two roles in the cell: 1) recruiting Shelterin to the PML bodies so telomeres can recombine and lengthen or 2) SUMOylation of Shelterin already in the PML bodies to maintain the telomeres. The role of SUMOylation of Shelterin proteins has yet to be explored in cells not undergoing ALT telomeric recombination, and the effects demonstrated by Potts and Yu (2007) have not been identified in telomerase positive cells (Potts and Yu, 2007; Zhao and Blobel, 2005; Xhemalce, *et al.*, 2007).

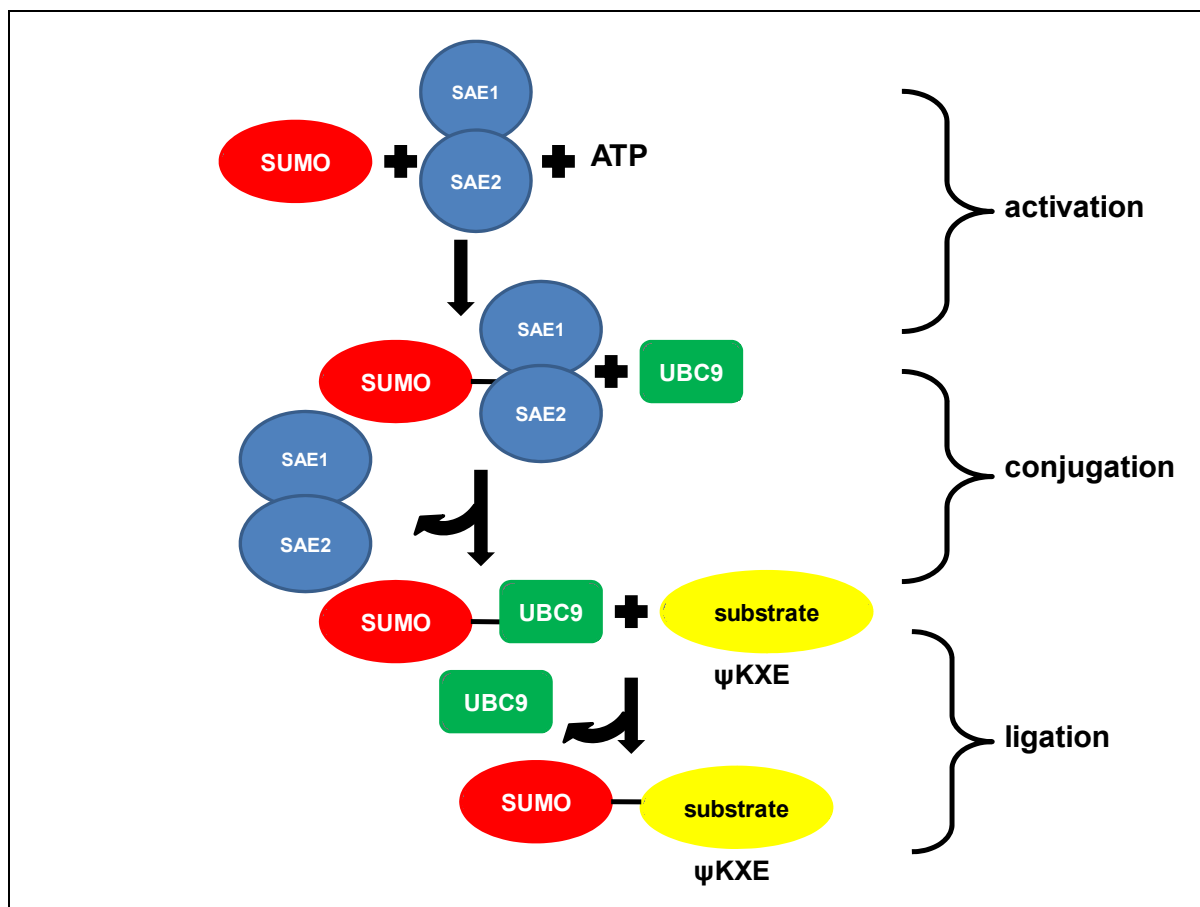


Figure 6. The SUMOylation Pathway. In an ATP-dependent step, SUMO is activated by binding to the E1-activating enzymes SAE1/2. SUMO is transferred to the E2-conjugating enzyme Ubc9. SUMO is then ligated to the substrate protein through an isopeptide bond formed between its C-terminal glycine and the ϵ -amino group of a lysine on the substrate protein (within the consensus sequence ψ KXE where ψ is a large hydrophobic residue, X is any amino acid). SUMO is processed by proteases termed SUMO-proteases or sentrin-specific proteases (SENPs). Adapted from: Verger, *et al.*, 2003.

Ku70 is another SUMOylated telomeric protein. Ku70 maintains telomeres by binding telomeric sequence and facilitating telomerase-mediated elongation in response to double strand breaks. Ku70 is transiently SUMOylated, which possibly results in increased protein levels through stabilization of the protein by SUMO. This is associated with overexpression of SUMO1 or SUMO2 in the cell, not necessarily with increased SUMOylation of Ku70 itself (although a SUMOylated version of Ku70 is observed). SUMO may actually act on an ubiquitin ligase, preventing its ability to degrade Ku70 and thus stabilizing Ku70 levels, although this hypothesis has yet to be fully explored (Yurchenko, *et al.*, 2007).

CHAPERONES

Heat shock proteins (Hsps), or chaperone proteins, are ubiquitous proteins required for cell processes including folding of the nascent polypeptide chains, preventing protein aggregation, and transporting proteins across membranes (Hartl, 1996). They were first identified as prominent proteins expressed under higher temperatures, hence the heat shock name (Lindquist and Craig, 1988). Other cellular stresses also induce their expression, including exposure to heavy metals, oxidative stress and inflammation, leading to the alternative name stress proteins (Jolly and Morimoto, 2000). The heat shock proteins include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsps ranging from 20-25 kDa, most of which tend to be localized to the cytosol, ER, and mitochondria in normal cells. In tumor cells, chaperone expression is often elevated presumably to maintain homeostasis and to allow tumor cells to tolerate microenvironment alterations. This results in normally lethal mutations promoting oncogenesis (Whitesell, *et al.*, 1994). One specific chaperone involved in this process is Hsp90, which has been shown to be overexpressed in breast cancer (along with Hsp70) and correlates

with poor prognosis (Grem, *et al.*, 2005), making it an ideal target for therapeutic cancer treatment.

Chaperones have been identified in telomere biology through their association with telomerase. The Hsp90 chaperone complex (including Hsp70, Hsp40, HOP, and p23) is necessary for assembly of active telomerase and inhibition of Hsp90 blocks telomerase assembly (Figure 7) (Holt, *et al.*, 1999). Additionally, continuous chronic inhibition of Hsp90 with the drug Radicicol results in telomere shortening of approximately 2.2kb over 2 months (Compton, *et al.*, 2006). Because the telomere associated proteins are so important to maintaining genetic stability, an interaction with a stress protein such as Hsp90 may indicate that there either is a problem at chromosome ends or constant modulation of protein structure and function is necessary at the telomere.

Hsp90

Hsp90 is one of the most abundant proteins in a normal, unstressed cell, making up 1-2% of the total soluble cytosolic proteins (Lai, *et al.*, 1984). In normal cells, Hsp90 is latent and uncomplexed. However, in tumor cells the Hsp90 chaperone is activated and complexed with co-chaperones (Figure 8) (Kamal, *et al.*, 2003). The Hsp90 family of chaperones has a variety of functions in the cell, including protein trafficking, refolding of proteins and degradation targeting of aggregated unstable proteins mediated by the proteasome (Nathan and Lindquist, 1995). Hsp90 activity is ATP dependent, and the N-terminal domain of Hsp90 contains a unique ATP-binding site known as the Bergerat fold that promotes client and co-chaperone binding as well as the development of specific inhibitors (Prodromou, *et al.*, 1997; Stebbins, *et al.*, 1997). The central region, known as the hinge region, allows for protein binding and regulatory functioning

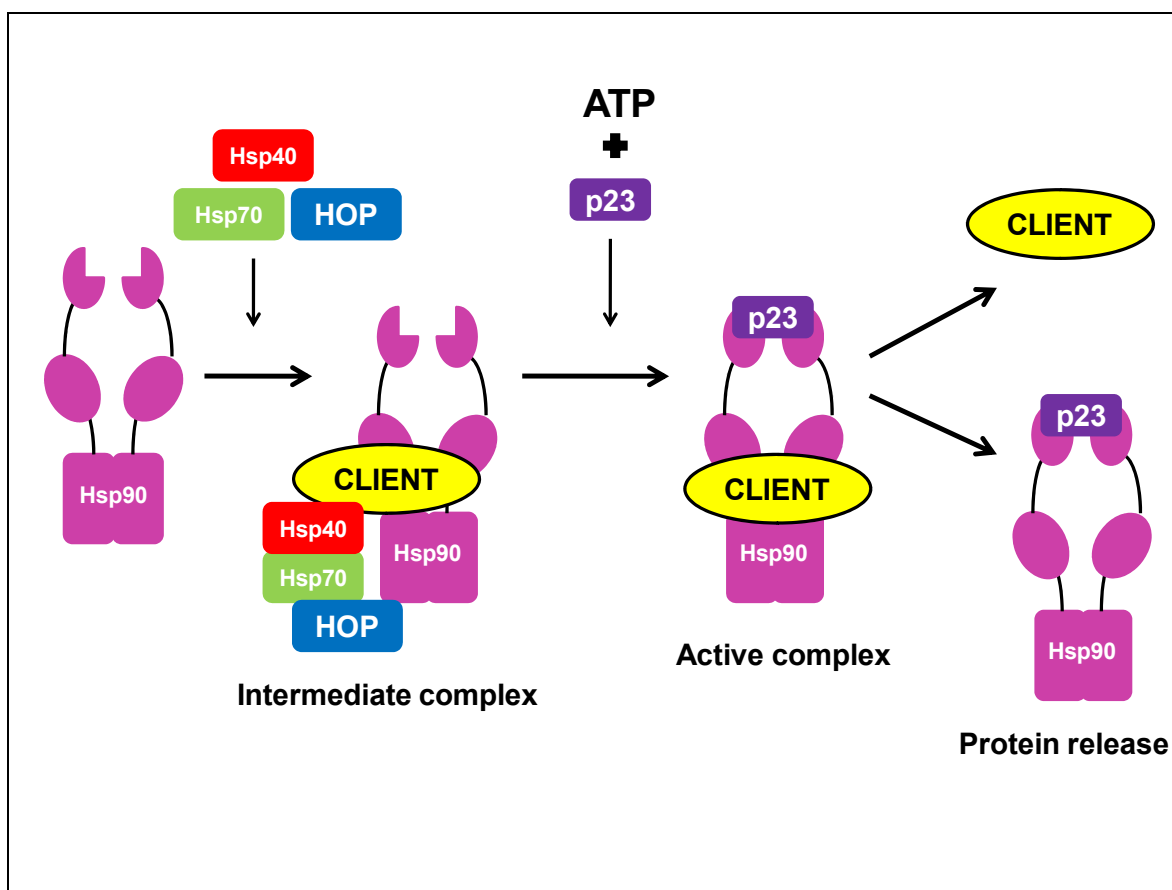


Figure 7. The Hsp90 Pathway. Hsp90 is associated with co-chaperones Hsp40, Hsp70, and HOP along with the client protein in its intermediate complex in its open conformation. In an ATP-dependent reaction, Hsp90 enters its active complex in a closed conformation, stably bound to the client protein, ADP and the co-chaperone p23. Finally, the mature client protein is released from the Hsp90/p23 complex. Adapted from: Kamal, *et al.*, 2003.

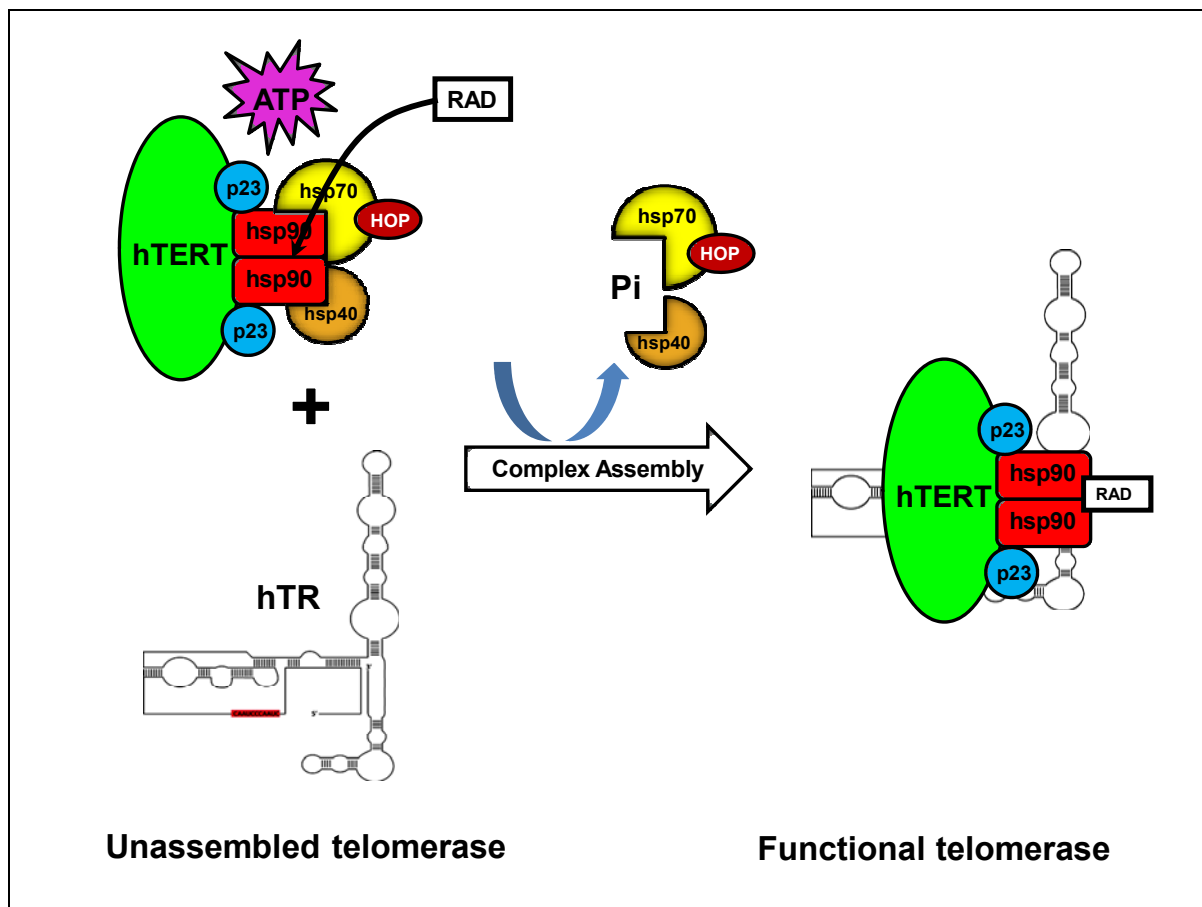


Figure 8. Active Telomerase Assembly. Unassembled telomerase exists in two complexes: the hTERT catalytic component bound to the chaperones Hsp90, Hsp70, p23, HOP and Hsp40 and the hTR RNA component. In an ATP dependent reaction, Hsp70, HOP and Hsp40 come off the hTERT component, which then stably associates with the hTR component forming active, functional telomerase. Hsp90 and p23 remain bound to telomerase. Radicol binds the ATP binding site of Hsp90, preventing it from properly binding the hTERT client protein. p23 is unable to remain associated to Hsp90 and hTERT is unable to bind correctly to hTR, resulting in non-functional telomerase assembly. Adapted from: Forsythe, *et al.*, 2001.

(Bogatcheva, *et al.*, 1999). The C-terminus primarily functions as the site for homodimerization (Minami, *et al.*, 1994).

Hsp90 exists in two isoforms: Hsp90 α and Hsp90 β . Hsp90 α is the more inducible and stable form (Lees-Miller and Anderson, 1989). Homodimerization increases after heat shock (or stress) and results in hydrophobicity (Minami, *et al.*, 1994). In stressed conditions, these dimers may associate into oligomers to promote Hsp90 binding to unfolded target proteins (Lanks, 1989).

As mentioned above, Hsp90, along with the co-chaperone p23, is required to assemble active telomerase through binding of the catalytic component, hTERT, and aids in assembly with the RNA component hTR (Holt, *et al.*, 1999). Hsp90 and p23 remain stably associated with active telomerase (Figure 8) (Forsythe, *et al.*, 2001). This association has been identified in prostate cancer with increasing levels of chaperone proteins correlating with cancer progression (Alkalin, *et al.*, 2001).

Hsp90 inhibitors

Various drugs have been developed to target Hsp90 and other chaperones by inhibiting the ability of chaperones to maintain their activation-competent conformation, resulting in the client proteins' degradation (Schulte, *et al.*, 1997). For example, 17-AAG binds to the complexed tumor specific form of Hsp90 (Kamal, *et al.*, 2003), thus inhibiting Hsp90 and inducing apoptosis (Sreedhar and Csernaly, 2004).

Two natural product inhibitors of Hsp90, geldanamycin and Radicicol, are used in both the laboratory and in clinical trials. Originally identified as an antimicrobial agent, geldanamycin

(GA) is in the ansamycin family (DeBoer, *et al.*, 1970). GA has antiproliferative activity in a range of tumor lines and reversibly binds the N-terminal ATP binding site of Hsp90 (Whitesell, *et al.*, 1994) to inhibit the ATPase activity of Hsp90 (Figure 9) (Prodromou, *et al.*, 1997). *In vivo*, geldanamycin treatment leads to hepatotoxicity (Supko, *et al.*, 1995), requiring the development of various, more tolerable, analogs. The most successful is 17-allylamino 17-demethoxy geldanamycin (17-AAG), which has decreased hepatotoxicity and increased Hsp90 inhibitory actions (Schnur, *et al.*, 1995). 17-AAG still exhibits solubility problems, whereas the analog 17-DMAG (17-NN-Dimethyl-Ethylene Diamine-Geldanamycin) may be used as an oral drug (Tian, *et al.*, 2004; Jez, *et al.*, 2003).

Radicalol (RAD) was originally isolated as an antifungal antibiotic (Delmotte and Delmotte-Plaque, 1953). Radicalol also acts on Hsp90 by binding the N-terminus and inhibiting its ATPase activity and is able to act in an antitumorigenic mechanism by preventing Hsp90 from folding client proteins (Figure 9) (Schulte, *et al.*, 1998). Radicalol is less cytotoxic than geldanamycin but does not contain the same tumor-cell specificity or solubility. *In vivo* studies have not resulted in significant antitumor activity (Geneg, *et al.*, 2004). However, the similarity to geldanamycin in its actions on Hsp90 and its similar effects at lower concentrations make it ideal for use in the laboratory. 17-AAG has completed Phase I testing as a single agent and is now in tumor specific Phase II trials and combination Phase I trials for breast and prostate tumors (Jameel, *et al.*, 1992). Hsp90 makes an attractive anti-cancer drug target for a variety of reasons: mutant proteins rely on Hsp90 for stabilization and prevention of degradation, and it has the potential to inhibit all six hallmarks of cancer through its client proteins. Those hallmarks (and examples of Hsp90 client proteins) are: ability to evade apoptosis (Survivin), angiogenesis (VEGF, VEGFR, and HIF1), immortalization (hTERT), tissue invasion and metastasis (MET

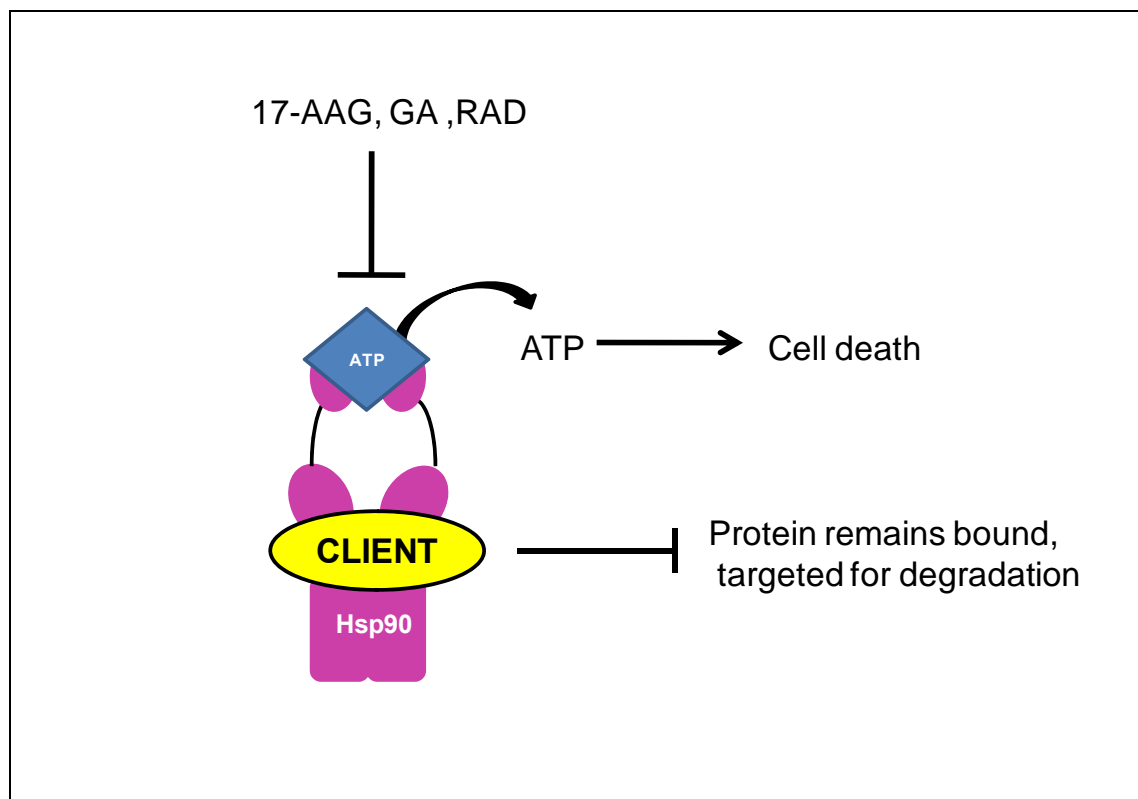


Figure 9. Hsp90 inhibitors. Inhibitors such as geldanamycin, 17-AAG, and Radicicol reversibly bind the N-terminal ATP binding site of Hsp90 and inhibit the ATPase activity of Hsp90 resulting in cell death. These inhibitors also prevent Hsp90 from releasing its client protein, thus targeting the protein for degradation. Adapted from: Schulte, *et al.*, 1997.

and MMP2), insensitivity to anti-growth signals (CDK4), and self-sufficiency in growth signals (RAF and ErbB2) (Hanahan and Weinberg, 2000; Kamal, *et al.*, 2003). In human tumor xenograft models, the complexed form of Hsp90 is more sensitive and has a greater affinity for specific inhibitors due to higher ATPase activity (Kamal, *et al.*, 2003). Therefore, 17-AAG accumulates in cancer cells specifically and synergizes with other cancer therapies. Hsp90 inhibitors attack multiple oncogenic pathways and prevent activation of alternative pathways, thereby preventing drug resistance (Jameel, *et al.*, 1994).

Hsp70

Hsp70 is a ubiquitous 70-kDa heat shock protein with multiple chaperoning activities including folding and assembly of nascent proteins, refolding of aggregated proteins, and cellular localization through interaction with client proteins in an ATP-dependent manner (Ryan and Pfanner, 2002; Pratt and Toft, 2003; Bukau, *et al.*, 2000; Hartl and Hayer-Hartl, 2002). Hsp70 is found to be required for assembly of active telomerase (Holt, *et al.*, 1999). Along with Hsp90, Hsp70 is upregulated in cancer (Ricanadiis, *et al.*, 2001). Mice with heat-inducible Hsp70 knocked out (Hsp70.1/3^{-/-}) displayed a higher rate of spontaneous genomic instability, with a noticeable effect on telomere stability. This suggests that Hsp70 not only plays a role in telomerase activity but also suppresses tumor formation with activity at the telomere (Hunt, *et al.* 2004).

Co-Chaperone, p23

Initially, p23 was identified as a ubiquitous protein complexed with Hsp90 and Hsp70 in avian progesterone receptors and was found to be a highly conserved acidic phosphoprotein

containing an aspartic acid-rich C-terminal domain (Johnson, *et al.*, 1994; Johnson and Toft, 1995; Johnson, *et al.*, 1996). As a co-chaperone of Hsp90, p23 binds to the ATP bound dimeric Hsp90 and stabilizes it in its ATP-hydrolyzed state (Richter, *et al.*, 2004; Sullivan, *et al.*, 2002; McLaughlin, *et al.*, 2002; Pratt and Toft, 1997). In steroid receptors, p23 increases the number of complexes that can bind to the hormone, again likely through stabilization of Hsp90 (Grenert, *et al.*, 1999; Young and Hartl, 2000). It has passive chaperoning activities of its own, suppressing aggregation of heat denatured citrate synthase and β -galactosidase (Bose, *et al.*, 1996; Freeman, *et al.*, 1996). Along with Hsp90, p23 is required to activate telomerase *in vitro* and remains stably associated with active telomerase (Holt, *et al.*, 1999; Forsythe, *et al.*, 2001). The yeast p23 ortholog, Sba1p, and the ortholog for Hsp90, Hsp82p, can modulate telomerase's ability to bind telomeric DNA both *in vitro* and *in vivo*, as well as being required for telomerase-mediated telomere extension in yeast (Toogun, *et al.*, 2007; Forafonov, *et al.*, 2008). Inhibition of Hsp90 also inhibits telomerase activity, suggesting that a fully functional Hsp90-p23 bound hTERT is required for telomerase to become active and extend telomeres (Keppler, *et al.*, 2006). Further roles of the Hsp90 complex (including p23 and Hsp70) in telomere biology have yet to be elucidated. Therefore, identification of chaperones at the telomere independent of telomerase, perhaps through interactions with other telomeric proteins, opens a new path to understanding telomere biology as well as possibly providing additional cancer targets through chaperone inhibition.

STUDY RATIONALE

The literature, along with work from our lab, suggests that the chaperones play a major role in telomere length regulation through their association with telomerase (Holt, *et al.*, 1999;

Akalin, *et al.*, 2001; Forsythe, *et al.*, 2001; Compton, *et al.*, 2006). However, there is no literature addressing further actions of the chaperones on the telomere independent of telomerase. Our identification of Hsp90, Hsp70 and p23's interactions with telomeric proteins reveals novel associations that may have significant implications in telomere biology. The interactions between Hsp90 and TRF2 and TRF1 are especially interesting, as both TRF1 and TRF2 are imperative for proper telomere maintenance. The focus of this work was to determine the mechanism of chaperone regulation on telomeres and the functional significance of the interactions of chaperones with telomere-associated proteins. In addition, a determination of the effects of chaperone down-regulation (pharmacologically) on telomeres was studied. Much has been studied in regards to telomeric protein-protein interactions and protein-DNA binding (rev. in Karlseder, *et al.*, 2003). It is possible that the chaperones facilitate these interactions, as they are known to have roles in protein-protein interactions and DNA binding (rev. in Hartl, 1996). Our specific aims were to 1) identify the interaction of chaperones Hsp90, Hsp70 and p23 with telomeric proteins TRF2, TRF1, TIN2 and TPP1 and 2) determine the functional significance of these interactions through pharmacologic inhibition of Hsp90. Our data indicates that Hsp90 does, in fact, associate with the telomere independent of telomerase, through novel interactions with the telomere binding proteins TRF2, TRF1, TIN2 and TPP1. To elucidate the mechanism for the interactions, we inhibited Hsp90 with compounds currently in clinical trials for breast cancer and examined the effects on telomere/protein binding, protein/protein interactions, protein degradation, and telomere dysfunction. The chaperone/telomere protein interactions may provide further understanding of telomere proteins and telomere protection, although this is beyond the scope of this study.

Chapter 2

Materials and Methods

Cell Culture

MCF7, a human breast cancer cell line, expressing ectopic hTERT was created as described previously (Elmore, *et al.*, 2002). MCF-7 cells were cultured using RPMI 1640 containing 5% fetal bovine serum and supplemented with gentamicin (0.05 mg/ml) grown at 37°C in 5% CO₂. H1299, a human lung carcinoma cell line, was cultured using DMEM containing 5% cosmic calf serum and supplemented with gentamicin (0.05mg/ml) grown at 37°C in 5% CO₂.

Reagents

Antibodies used include: anti-mouse Hsp90, anti-mouse Hsp70, and anti-mouse p23 (from Dr. David Toft, Mayo Clinic, Rochester, MN), anti-mouse Hsp90 (Stressgen, Ann Arbor, Michigan), anti-rabbit TRF2 (Imgenex, San Diego, CA), anti-rabbit TRF1 and anti-rabbit Ubiquitin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-mouse hTERT (Rockland, Gilbertsville, PA), anti-rabbit Pan-Sumo, anti-rabbit SUMO1, anti-rabbit SUMO2 (Abgent, San Diego, CA) and anti-mouse IgG (Sigma, St. Louis, MO).

Chromatin Immunoprecipitation

For ChIP, cells were grown to approximately 90% confluency and fixed with 1% formaldehyde directly on the plate. Glycine was added to a final concentration of 0.125M to

remove excess formaldehyde. Following a PBS wash, cells were resuspended in lysis buffer (5mM PIPES pH 8.0, 85mM KCl, 0.5% NP-40, 1mM PMSF, 1X protease inhibitors), incubated on ice and lysed by dounce homogenization. Nuclei were then lysed (50mM Tris pH 8.1, 10mM EDTA, 1% SDS, 1X protease inhibitors) and sonicated to ~1000 bp fragments using a Missonex 3000 sonicator with microtip. The sheared DNA was diluted 3:1 in 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl. After preclearing with 25% salmon sperm DNA/Protein A/G, the supernatants were equally divided and immunoprecipitated overnight at 4°C with antibodies directed against Hsp90 (1µg/ml), Hsp70 (1µg/ml), or p23 (1µg/ml), TRF2 (5µg/ml), hTERT (5µg/ml), IgG (0.5µg/ml) and TRF1 (5µg/ml). Immunocomplexes were recovered with a Protein A/G slurry and washed sequentially in Low Salt Immune Complex Wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl), High Salt Immune Complex Wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), LiCl Immune Complex Wash (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1) and 1X TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). Immune complexes were eluted with 1% SDS, 0.1M NaHCO₃. Crosslinks were reversed by overnight incubation in a 5M NaCl solution at 80°C. At the completion of this incubation, Proteinase K was added to the eluate in 0.5M EDTA and 1M Tris pH 6.5 at 45°C for 1h. DNA was then precipitated with ethanol, and the pellets resuspended in dH₂O. DNA samples were applied to a slot blot apparatus with a nylon membrane. The membrane was then processed and probed for a telomeric signal using a radiolabeled telomeric primer, as described previously (Elmore, *et al.*, 2002).

Transcription/Translation

TRF2 was synthesized in the RRL system (TNT, Promega) as described previously (Holt, *et al.*, 1999) in the presence of [³⁵S] methionine. A total of 1µg of plasmid DNA was used for each TnT reaction. The WT TRF2 plasmids were a gift from Dr. Dominique Broccoli (Memorial University Medical Center, Savannah GA).

Drug Treatments

Radicalol was tested at varying concentrations (Sigma, St. Louis, MO) and MG132 at 10µM (Sigma, St. Louis, MO) both solubilized in DMSO (control). *In vitro*, TnT lysates were treated with 50µg/ml Radicalol or Geldanamycin at 30°C for 90 minutes. Cell cultures were treated with 0.1µM or 0.3µM Radicalol for specified times at 37°C.

Growth Assay

H1299 cells were plated 100,000 cells/well in a 6-well dish. At 6 hours after plating, cells were treated with varying concentrations of Radicalol (0.03µM, 0.1µM, 0.3µM, 1µM, and 3µM, as well as DMSO vehicle treated). Drug was replaced every 48 hours. Cells from triplicate cultures were trypsinized and counted using a hemocytometer at 24 hrs, 72 hrs, and 120 hours and presented as total cell numbers.

Cell Extracts

For whole cell extracts, cells were pelleted and resuspended in Modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 0.25% sodium-deoxycholate, 1mM EDTA) plus

1 mM protease inhibitor cocktail (Calbiotech). Cells were lysed by sonication on ice at 60% power for 10 seconds four times using a Missonex 3000 sonicator with microtip. For nuclear extracts, nuclei were isolated using the Cell Lytic NuCLEAR Extraction kit without detergent (Sigma Chemical Companies). The cells were lysed in a hypotonic lysis buffer and dounce homogenized. The extraction buffer was diluted for a final NaCl concentration of 150mM with 1X Dilution and Equilibration Buffer to ensure sufficient lysis of the nuclei without disruption of interactions.

Immunoprecipitation

For immunoprecipitations from *in vitro* assembly reactions, Hsp90, Hsp70, p23, TRF2 and IgG (antibodies at 1µg/ml) antibodies were added to a 50/50 mixture of Protein A/G beads (Roche) and incubated on ice for two hours. Lysates from the TRF2 TnT reaction were then added to the antigen/bead complex and incubated at 4°C rotating overnight. Beads were then pelleted and washed four times in CHAPS lysis buffer (19mM Tris-HCl pH7.5, 1mM MgCl₂, 0.1mM Benzamidine, 1mM EGTA, 5mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol).

For immunoprecipitation reactions from nuclear and whole cell extractions, antibodies Hsp90 (0.5µg/ml), Hsp70 (0.5µg/ml), p23 (0.5µg/ml), TRF2 (1µg/ml), hTERT (1µg/ml), Ubiquitin (1µg/ml) and SUMO1/2 or Pan-SUMO (1µg/ml) were added to corresponding samples and incubated on ice two hours. Prewashed Protein A/G beads were then added to the samples and incubated overnight 4°C, with continual agitation. Following the incubation, beads were pelleted and washed three times with CHAPS lysis buffer.

Gel Electrophoresis and Imaging

For both immunoprecipitations from TnT reactions and nuclear extractions, Laemmli buffer was added to beads, which were then heat denatured at 95°C for 10 minutes. Samples were separated by SDS-PAGE. For the nuclear extractions, following the electrotransfer of proteins onto Hybond nitrocellulose membranes (Amersham, Arlington Heights, IL), a standard Western blotting protocol was performed (Elmore, *et al.*, 2002). For TNT reactions, after samples were run on SDS-PAGE, the gel was fixed in a 10:20:70 solution of glacial acetic acid:methanol:water. The gel was then dried and exposed to a phosphoimager. Images were visualized using the PhosphoImager (Molecular Dynamics, Sunnyvale, CA) or the Typhoon Variable Mode Imager (GE Healthcare Life Sciences, Piscataway, NJ) and ImageQuant (GE Healthcare Life Sciences, Piscataway, NJ) image analysis software. Densitometry was performed using the ChemiImager software (Alpha Innotech, San Leandro, CA).

Immunofluorescence

Cells were plated on alcohol, heat-treated cover slips (22mm) in a 6-well dish and grown to approximately 70% confluency. Cells were then treated with 0.3 μ M Radicicol for 1, 2, 4, 6, 8 and 16 hrs as indicated. Following the appropriate incubation, the cells on the cover slips were fixed with 3.7% paraformaldehyde in 1X PBS, washed with 1X PBS and permeabilized with 0.5% NP-40 in 1X PBS, and washed with 1X PBS. Cover slips were then blocked with PBG buffer (0.2% cold water fish gelatin, 0.5% BSA in 1X PBS), and incubated in rTRF2, rTRF1 (5 μ g/ml in PBG) or mHsp90 (0.5 μ g/ml in PBG) antibody at 4°C overnight. Cover slips were washed in 1X PBS and then incubated with either Alexa 568 anti-rabbit or Alexa 488 anti-mouse

secondary antibody (Invitrogen, Carlsbad, CA). Cover slips were washed with 1X PBS, and stained with 4',6-diamidino-2-phenylindole (DAPI) followed by mounting in Vectashield (Vector Lab). Secondary antibody only cover slips served as negative controls. Representative images were captured using either a Nikon Eclipse E800M fluorescence microscope or a Leica TCS-SP2 AOBS confocal laser scanning microscope. Images were analyzed using the Leica LCS Lite masked cytofluorogram confocal software.

Statistical Analysis

Densitometry was performed using the ChemiImager software (Alpha Innotech, San Leandro, CA). For *in vitro* immunoprecipitations, western blots, and immunofluorescence analyses, a Duncan's new multiple range test was performed to assess statistical significance of mean sample differences with significance set at $p < 0.05$ from three independent experiments. For *in vitro* Immunoprecipitation to compare drug treatments, ANOVA was performed with significance set at $F < 0.05$.

Telomere Length Analysis

Telomere length was determined using the terminal restriction fragment (TRF) length assay as described previously (Elmore, *et al.*, 2002). Briefly, DNA was isolated from cells using genomic tips (Qiagen). A total of 7 μ g of genomic DNA was digested with a cocktail of restriction enzymes (AluI, HaeIII, HinfI, MspI, and RsaI, New England Biolabs) and resolved on a 0.7% agarose gel. A G-rich telomeric probe [(TTAGGG)₄] was labeled through random priming with [γ -³²P]ATP (6000 Ci/mmol), with unincorporated nucleotides being removed using a QIAquick nucleotide removal kit, according to manufacturer's protocol (Qiagen). The gel

was dried and hybridized with the radiolabeled G-rich probe for 12-16 hours. Following washing to remove unbound probe, the gel was exposed to a PhosphorImager cassette overnight (Molecular Dynamics).

Cytogenetics

To determine if telomeric sequences were involved in end-to-end fusions, metaphase spreads from H1299 cells were scored following fluorescence in-situ hybridization (FISH) using a pantelomeric peptide nucleic acid probe (PNA). The metaphase spreads from the treated (1 day; 3 days; and 5 days chronic exposure to a 0.3 μ M solution of Radicol) and control (untreated) cultures were harvested using standard procedures (Rooney and Czepulkowski, 1992). Briefly, actively dividing cells were blocked in mitosis through a 1 hour exposure to 0.1 μ g/ml of colcemid. Following lifting (using trypsin), the cells were incubated in a hypotonic solution (0.075M KCl at 37°C for 20 minutes). The cells were then fixed, using serial room temperature incubations, in Carnoy's fixative (3 parts methanol to 1 part acetic acid) and slides made using standard procedures. The FISH was performed using a synthetic pantelomeric probe following the manufacturer's protocol (DAKO). Briefly, after fixation, pre-treatment, and dehydration, the metaphase chromosomes and FITC-labeled probe [(C₃TA₂)₃] were co-denatured (5 min at 80°C). Following hybridization (30 minutes at room temperature), the excess, unbound probe was removed by washing (65°C for 5 minutes with agitation). The slides were then dehydrated with cold ethanol, air dried, and counterstained with a DAPI/Antifade solution (Cytocell).

The presence or absence of telomeric signals or end-to-end chromosomal fusions was scored using a Zeiss Axioskop, with representative images being captured using a Cytovision

image analysis system (Applied Imaging). The frequency of chromosomal findings present in the cell treatments and control cultures were compared using a Chi-square goodness of fit test, with an α level of 0.05.

Prediction of SUMOylation sites

Potential TRF2 SUMOylation sites were identified using the Abgent SUMO PLOT program (Abgent, San Diego, CA).

In vitro SUMOylation assay

Using the Vaxron *In Vitro* SUMOylation Assay kit (Vaxron, Rockaway, NJ) TRF2 or dnTRF2 was synthesized using the RRL system and radiolabeled with ^{35}S (TNT, Promega). A 10X Reaction Buffer (0.2M HEPES-NaOH, pH 7.4, 50mM MgCl_2 , 20mM ATP, 0.5mM PMSF, 1mg/ml BSA), SUMO-1 (1mg/ml), GST-Ubc9 (0.4mg/ml), and SAE1/2 (1 unit/ μl) from the kit were then added to the TnT lysates (10 μl), which were incubated at 37°C for 120 minutes. Samples were separated on an SDS-PAGE gel and exposed to a PhosphorImager cassette overnight (Molecular Dynamics).

Site-Directed mutagenesis

Mutations were created using the QuikChangeII Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For single mutant strand synthesis K150R, the primers used were KR150TRF2S (GTGCTGGAGATGATTAGAACGGAATTTACAACCTGACAGAAGC AGTGG) and KR150TRF2AS (CCACTGCTTCTGTCTCAGTGTAATTCGGTTCTA ATCATCTCCAGCAC). For the K255R mutation, primer KR255TRF2S (CGATGG

CCAAAAAGGCTTTGAGATCTGAGTCCGCTGCC) and KR255TRF2AS (GGCA GCGGACTCAGCATCTCAAAGCCTTTTTGGCCATCG) were used. A final concentration of 10ng of pcDNA3TRF2full dsDNA was added to the PCR reaction (125ng S and AS primer, 10x PCR buffer, dNTP, 5% DMSO and 1µl PfuUltra HF DNA Polymerase; cycling conditions: 95°C 30 sec, 18 cycles 95°C 30 seconds, 55°C 1 minute, 68°C 7 minutes). The reaction was then digested for 1 hour at 37°C with 1µl DpnI (10 units/µl) enzyme, transformed into DH5α competent cells (Bioline, Taunton, MA), and screened by sequence analysis across the mutation region. A single positive clone was sequence verified across the entire insert region. For the double mutant, K150R was used as a template for the K150R/K255R double mutation. Reaction and verification were performed as above, with DpnI digestion, transformation and sequence analysis.

Chapter 3

Chaperone-mediated Regulation of Telomere Associated Proteins

INTRODUCTION

The telomeric multiprotein complex organizes the linear chromosome end into a t-loop structure, which contributes to telomere length regulation and end protection (Griffith, *et al.*, 1999). The t-loop prevents the telomere from being recognized as damaged DNA and from cellular exonucleases acting on the telomere (Griffith, *et al.*, 1999). One protein in particular, TRF2, aids in protection of telomeres from end-end fusions by directing the invasion of the 3' single strand overhang into duplex telomeric DNA, creating the t-loop (Griffith, *et al.*, 1999; Stansel, *et al.*, 2001). TRF1 is another protective telomere binding protein that coats the double stranded telomeric DNA and aids in formation of the t-loop. In addition to the structural function of TRF1 at the telomere, its main role in telomere length homeostasis has been reported to be preventing telomerase access in normal cells (Smorgorzewska, *et al.*, 2000). As a second major player in protection of the telomere, it is imperative to understand TRF1's regulation and post-translation modifications, as well as TRF2.

Heat shock proteins (Hsps), or chaperones, are ubiquitous proteins required for cell processes including folding of the nascent polypeptide chains, preventing protein aggregation, and transporting proteins across the membrane (Hartl, *et al.*, 1996). In tumor cells, chaperone expression is often increased, presumably to maintain homeostasis and to allow tumor cells to tolerate microenvironment alterations, which results in normally lethal mutations enhancing oncogenesis (Sausville, *et al.*, 2003). One specific chaperone involved in this process is Hsp90,

which is over-expressed in breast cancer (along with Hsp70) and correlates with a poor prognosis (Grem, *et al.*, 2005), making it a potential target for therapeutic treatment of cancer.

We hypothesized that the interaction of chaperones and telomere proteins occurs as a means for Hsp90 to target TRF1 and TRF2 for degradation. Hsp90 acts on mutated proteins in tumor cells to target them for degradation, and because many proteins bound to the telomere also tend to be altered in tumor cells (either from critically short telomeres or deregulation/modification), it is likely that Hsp90 is targeting telomere-associated proteins for ubiquitination as well. The specific aims of this study were to attempt to define the role of chaperones at the telomere by first defining the interactions between Hsp90 and the telomeric proteins TRF1 and TRF2 through Co-IP/Western and then determining the functional significance of the interactions through pharmacologic inhibition of Hsp90 using Co-IP/Western and colocalization studies. e of the interaction at the present time, however further studies are being conducted/

Chaperones Bind to the Telomere

Previous studies have demonstrated that Hsp90 associates with the telomere through a direct interaction with hTERT (Holt, *et al.*, 1999; Forsythe, *et al.*, 2001). We hypothesized that Hsp90 and perhaps other components of the Hsp90 chaperone complex are present at the telomere independent of telomerase. To determine if chaperones bind the telomere, a chromatin immunoprecipitation (ChIP) assay was performed. We used H1299 lung carcinoma cells, which have naturally long telomeres (approximately 25 kb), providing plenty of substrate. In addition to H1299 cells, MCF7 breast carcinoma cells and normal BJ foreskin fibroblast cells, both ectopically overexpressing hTERT, were used because expression of hTERT elongates telomeres, providing more substrate for telomere associated proteins to bind and thus increasing

the sensitivity and detection of these associations. As it is difficult to design PCR primers for telomeric repeat sequences, we radiolabeled a telomere sequence specific probe to visualize telomeric DNA binding of our immunoprecipitated fragments. Positive signal in the Chromatin Immunoprecipitation assay (ChIP) indicates that a specific protein was bound directly to telomeric DNA. TRF1 and TRF2 serve as positive controls as they are known to bind directly to the telomere. IgG serves as a negative control. We show that Hsp90 and Hsp70 bind to telomeres in the cancer cell lines H1299 and MCF7 hTERT (Figure 10A and 10B). The normal BJ-hTERT foreskin fibroblast cells also demonstrate that the chaperone Hsp70 was found at the telomeres in normal cell lines (Figure 10C). Interestingly, we only detect telomeric p23 above background IgG levels in the BJ-hTERT cells (Figure 10C) and not in the H1299 or MCF7 cells (Figure 10A and 10B), which may be due to p23's association with Hsp90 and indirect association with telomeric proteins in cancer cells. Previous studies from our laboratory indicate that Hsp90 and p23 remain stably associated with the telomerase enzyme (Forsythe, *et al.*, 2001), indicating that a subfraction of these chaperones are likely associated with hTERT at the telomere. However, based on the amount of Hsp90 and Hsp70 (each at 1 μ g/ml) bound at the telomere (Figure 10A-10C), their association is likely, at least in part, independent of hTERT. In the H1299 cells, hTERT was present at similar levels to all other proteins, likely due to the increased amount of telomere substrate (Figure 10A).

Interaction of Telomere Associated Proteins and Chaperones

Since a subfraction of Hsp90 binds to the telomere, possibly independent of hTERT, we hypothesized that components of the Hsp90 chaperone complex associate with telomere proteins, including TRF1 and TRF2. The rabbit reticulocyte lysate (RRL) transcription and translation

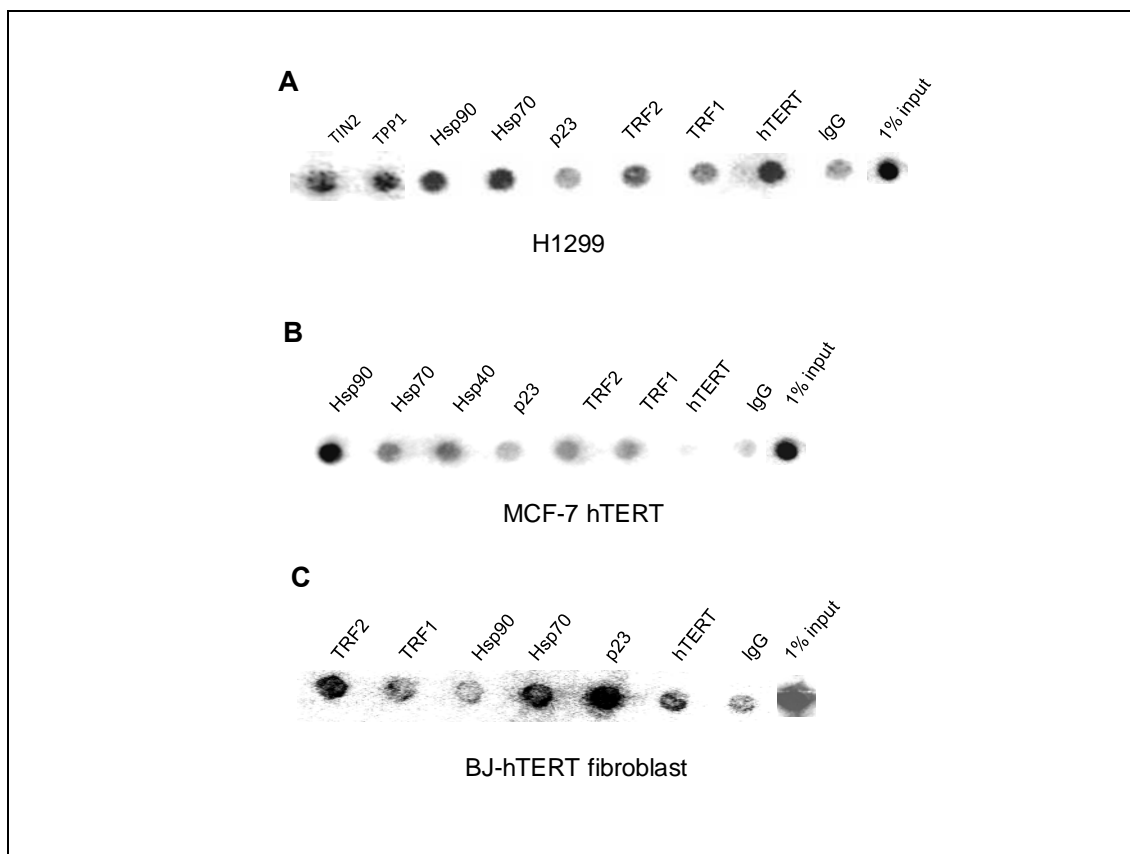


Figure 10. Chaperones are present at telomeres. Using chromatin immunoprecipitation and a telomeric probe, antibodies for the indicated proteins were used to determine if there was an interaction with telomere sequences. **A.** H1299 cells. IgG serves as a negative control. Input is 1% sheared DNA and serves as the positive control for hybridization. TRF1 and TRF2 serve as positive controls, since they are known to bind directly to the telomere (Broccoli, *et al.*, 1997) **B.** MCF7 cells overexpressing hTERT and **C.** BJ-hTERT fibroblast cells. The chaperones and telomeric proteins are bound to the telomeric DNA in all three cell lines.

(TnT) system is ideal for identifying interactions with chaperone proteins, as there is an abundance of chaperone proteins in the reticulocyte lysate. When the protein of interest, such as TRF2, is synthesized, it is the only radiolabeled protein present in the system to interact with the chaperones. Using this TnT system, we can therefore determine if TRF2 directly interacts with the chaperone proteins independent of any other protein in the cell. Thus, TRF2 was radiolabeled and synthesized *in vitro*, and the resulting lysate was immunoprecipitated for Hsp90, Hsp70 and p23. When the immunoprecipitates were visualized using an SDS-PAGE gel, positive bands for TRF2 indicated a direct interaction with Hsp90, Hsp70, and p23 *in vitro* (Figure 11).

To further explore the hypothesis that Hsp90 interacts with telomeric proteins, we extended our studies to co-immunoprecipitation (Co-IP) in cultured MCF7-hTERT cells and H1299 cells to assess whether the *in vitro* interaction between telomeric proteins and Hsp90 exists in tumor cells. To ensure specificity and increase sensitivity in our cell studies, all subsequent studies were done using nuclear extracts (unless otherwise indicated). Hsp90 is primarily an abundant cytoplasmic protein making up 1-2% of total cytoplasmic protein while telomeric proteins are strictly nuclear (Lai, *et al.*, 1984). Therefore, nuclear extracts increase the likelihood that the interaction between TRF2 and Hsp90 is a real interaction and not an artifact of cytoplasmic Hsp90 contamination. Figure 12A suggests a high purity of our nuclear extraction, through Western blot showing Histone H1, a nuclear protein, present only in nuclear extract lysates. Co-IP/Western blots show that both TRF2 and TRF1 interact with Hsp90 and Hsp70 in the H1299 cell line (Figure 12B and 12C, respectively) and TRF2 interacts with Hsp90 and Hsp70 in the MCF7-hTERT cell line (Figure 13A and 13B). There was no detectable p23/TRF2 interaction in MCF7-hTERT or H1299 cells as was seen *in vitro*, which may suggest that p23 is associating with TRF2 through other telomere binding or chaperone proteins.

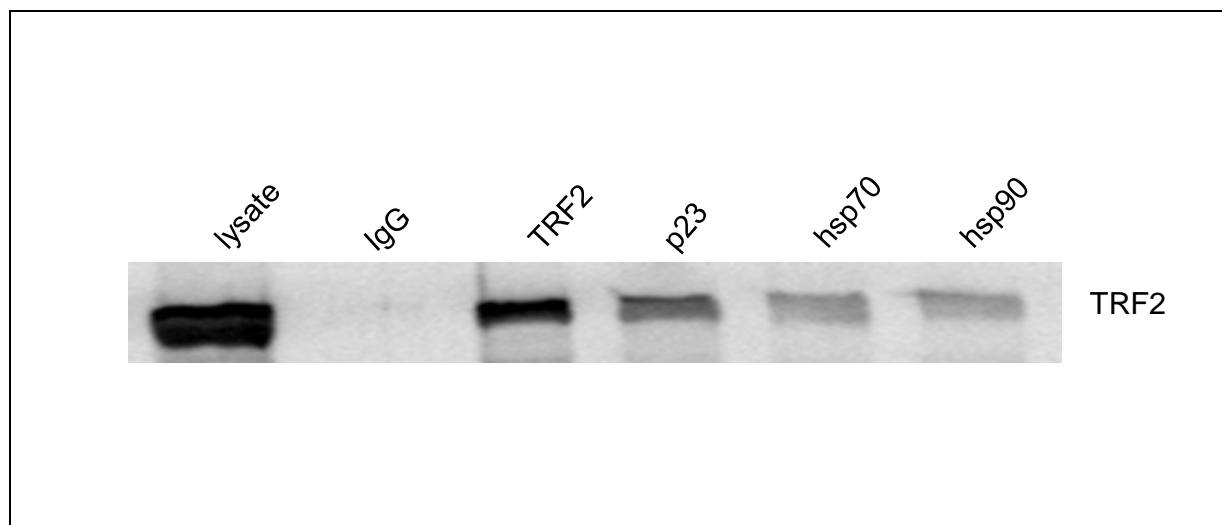


Figure 11. *In vitro* interaction of TRF2 with chaperones. Immunoprecipitation was performed using antibodies against TRF2, Hsp90, Hsp70, and p23 after expression of ^{35}S -methionine labeled TRF2 in the TnT system. A positive interaction result shows precipitation of the radiolabeled TRF2. 5 μl lysate was loaded as a positive control, and IgG serves as a negative control.

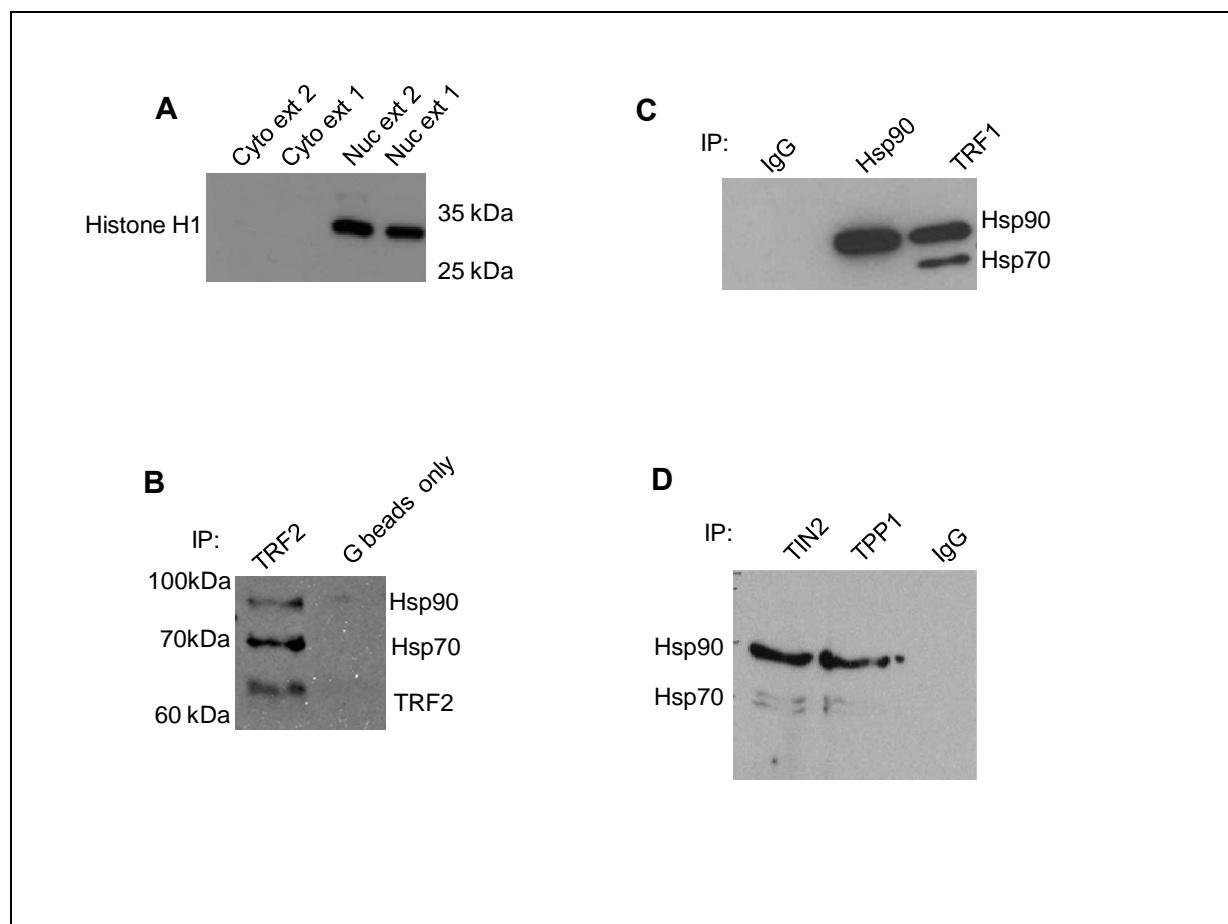


Figure 12. Interaction of telomeric proteins with chaperones from H1299 cells. **A.** Verification of nuclear extracts. Cells were lysed, and nuclear and cytoplasmic fractions were isolated. A Western blot using 20 μ g of protein from each extract of two separate samples was probed for the nuclear protein, Histone H1, which runs at 32 kDa. **B.** TRF2 was immunoprecipitated from nuclear extracts and a Western blot was probed for Hsp90, Hsp70 and TRF2. Protein G agarose beads only serve as a negative control. **C.** Western analysis of immunoprecipitations of the telomere binding protein TRF1 was accomplished using chaperone, TRF1 and IgG antibodies. TRF1, Hsp90 and IgG were immunoprecipitated from nuclear extracts as indicated and a Western blot was probed for Hsp90 and Hsp70. IgG serves as a negative control. **D.** Western analysis of immunoprecipitations of telomere-associated proteins was accomplished using chaperone, TIN2, TPP1 and IgG antibodies. TIN2 and TPP1 were immunoprecipitated from nuclear extracts and a Western blot was probed for Hsp90 and Hsp70. IgG serves as a negative control.

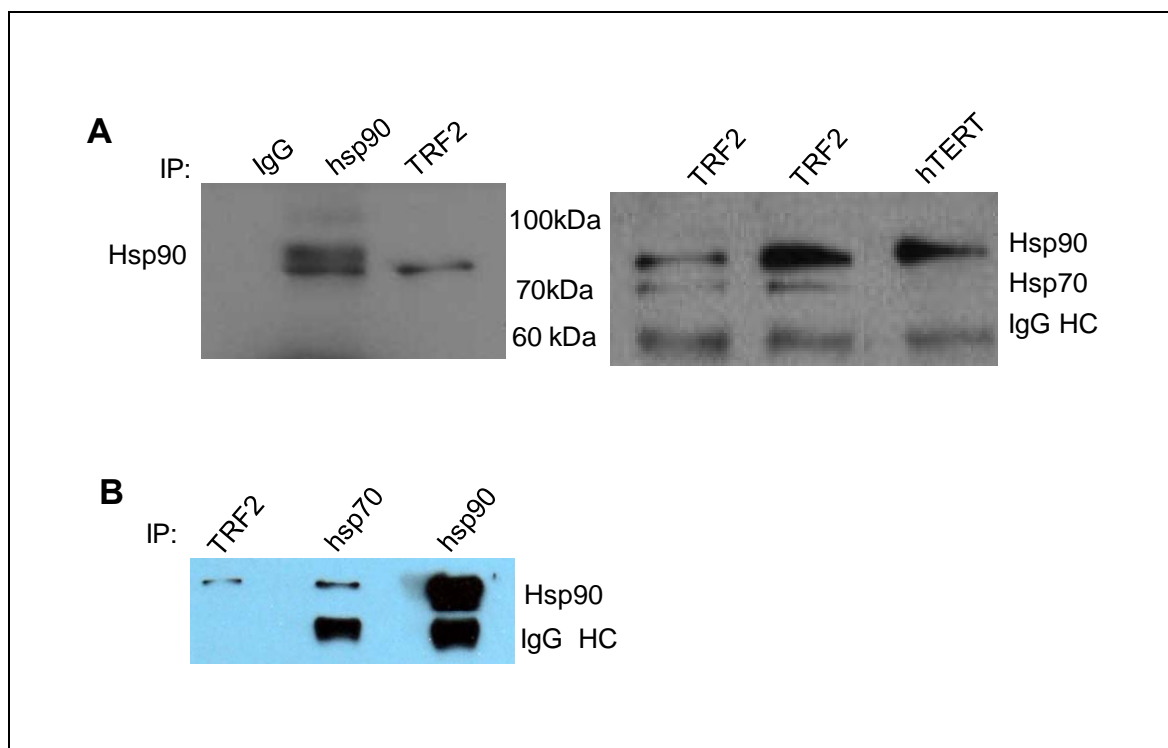


Figure 13. Interaction of TRF2 with chaperones from MCF7-hTERT cells. **A.** Western analysis of immunoprecipitations of telomere-associated proteins was accomplished using Hsp90, TRF2 and IgG antibodies. TRF2 and Hsp90 were immunoprecipitated from nuclear extracts and a Western blot was probed for Hsp90. IgG serves as a negative control (left panel). TRF2 and hTERT were immunoprecipitated and a Western blot was probed for Hsp90 and Hsp70. hTERT serves as a positive control for immunoprecipitation and Western blot because of its known interaction with Hsp90 (right panel). IgG Heavy Chain (HC) serves as antibody controls and are visualized when the primary and secondary antibodies are derived from the same species. **B.** Hsp90 Western blot after immunoprecipitation with Hsp90, Hsp70 and TRF2. Hsp90 and Hsp70 are known to interact, and an interaction between Hsp90 and TRF2 was observed.

The interaction between TRF2, TRF1 and Hsp90/Hsp70 led us to hypothesize that Hsp90 and Hsp70 were also interacting with other telomere associated proteins, such as TPP1 and TIN2. TIN2 is one of the six Shelterin proteins and forms a “bridge” between TRF2 and TRF1 (Ye, *et al.*, 2004) and interacts directly with TRF1, mediating its activity and acting as a negative regulator of telomere elongation (Kim, *et al.*, 1999). Another member of the Shelterin complex, TPP1, binds POT1 and directly interacts with TIN2 (Ye, *et al.*, 2004). A Co-IP/Western of TIN2 and TPP1 probed for Hsp90 and Hsp70 verifies that both proteins interact with both chaperones in H1299 cells (Figure 12D), although the association with Hsp70 for both TIN2 and TPP1 is fairly weak.

Through Co-IP/Western we have identified novel interactions of the telomeric proteins TRF1, TRF2, TIN2 and TPP1 with the chaperones Hsp90 and Hsp70. These interactions suggest that the chaperones may be contributing some regulatory function on telomeric proteins such as mediating protein-protein interactions of telomeric proteins or facilitating association with the telomere.

Inhibition of Hsp90

The interaction between TRF2/TRF1 and the Hsp90 chaperone suggests a functional relationship between the proteins. We hypothesized that inhibition of Hsp90 would result in a disruption in TRF1/2's ability to bind to the telomere resulting in degradation of each protein, thereby disrupting their protective function at the telomere. Inhibitors of Hsp90 are currently used in breast cancer therapy, notably 17-AAG (an analog to Geldanamycin), which is in Phase II clinical trials (Banjeri, *et al.*, 2005; Goetz, *et al.*, 2005; Grem, *et al.*, 2005). We used both Geldanamycin (GA) and Radicicol (RAD) in *in vitro* experiments and RAD in cell culture

experiments to determine if inhibition of Hsp90 affects its interaction with TRF2 and TRF1, as well as their function at the telomere. We used the RRL system to synthesize TRF2 or TRF1 *in vitro* and then added 50 $\mu\text{g}/\text{ml}$ of Radicicol, Geldanamycin or equivalent volumes of DMSO as the untreated sample and performed an assembly reaction at 30°C for 90 minutes. The TRF2/TRF1 that was assembled in presence of drug was immunoprecipitated with antibodies against Hsp90, TRF2, or TRF1 (Figure 14A and C). We first performed an ANOVA to determine if there was a significant difference between the effects of the two drugs. There was no significant difference when RAD is compared to GA for each immunoprecipitation ($F > 0.05$). However, using the Duncan's new multiple range test to compare each drug treatment for each IP, the Hsp90/TRF2 interaction, when treated with either RAD or GA, is significantly disrupted when compared to the DMSO control for RAD only ($p < 0.05$) (Figure 14B). Thus, although there is no difference in disruption of interaction between the two drugs, there is a difference between RAD and untreated. For this reason and reduced cellular toxicity, we continued using RAD rather than GA in subsequent cell based experiments.

A growth curve was performed to determine the optimal RAD concentration to use in our experiments. 0.3 μM RAD had an effect on cell count without causing total cell death at five days. At 1 μM and higher, cells began to die at day 3, and at 0.1 μM and lower, minimal effect was observed (Figure 15). Therefore, 0.3 μM RAD was used in subsequent experiments.

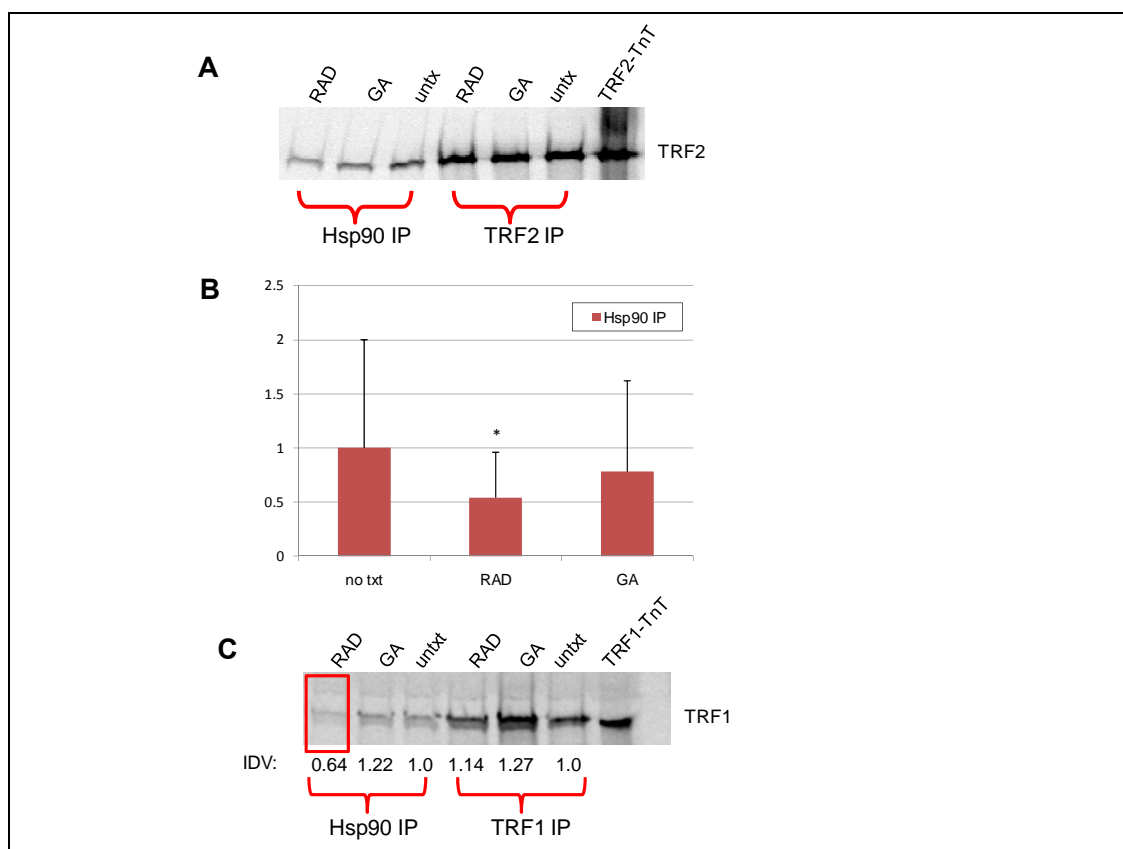


Figure 14. Hsp90 inhibition affects TRF2/Hsp90 interaction. **A.** TRF2 was synthesized *in vitro* and then 50 μ g/ml Radicicol, Geldanamycin, or DMSO was added to the synthesized TRF2 assembly reaction. Samples were then immunoprecipitated for Hsp90 or TRF2. 3 μ l of synthesized TRF2-TnT was run as a positive control. **B.** IDV (integrated density values) were normalized to untreated samples (value = 1) for each immunoprecipitation. Duncan's new multiple range test suggests a significant difference between the Hsp90/TRF2 interaction when treated with RAD compared to untreated from three independent experiments ($p < 0.05$). **C.** TRF1 was synthesized *in vitro* and then 50 μ g/ml Radicicol, Geldanamycin, or DMSO was added to the synthesized TRF1 assembly reaction. Samples were then immunoprecipitated for Hsp90, and TRF1. The greatest interference in the Hsp90/TRF1 interaction was seen with Radicicol treatment as identified through quantitation with densitometry (see outlined box) from one experiment. IDV (integrated density values) were normalized to untreated samples for each IP (value = 1). 3 μ l of synthesized TRF1-TnT was run as a positive control.

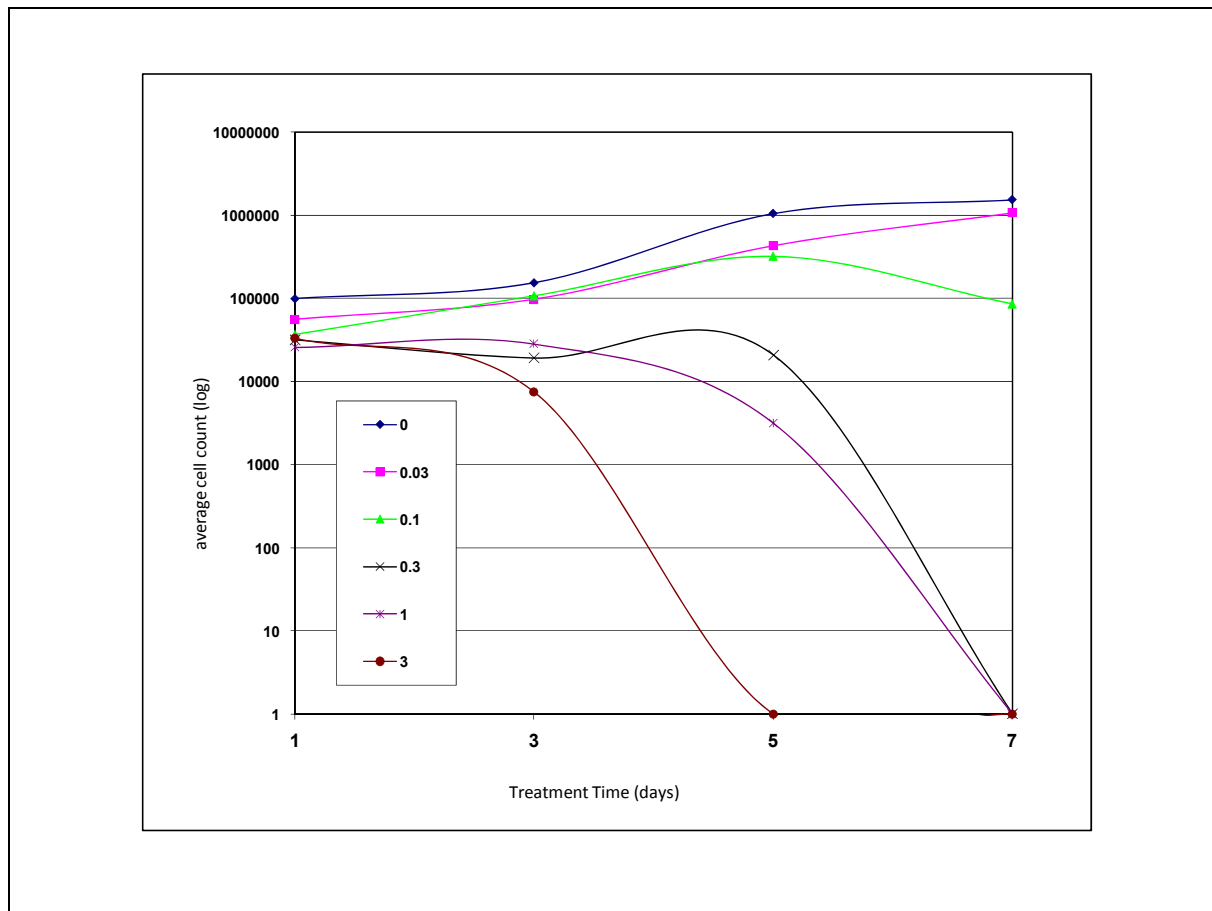


Figure 15. Growth curve of H1299 cells after Radicicol treatment. Cells were treated with varying concentrations over 7 days to determine optimal drug treatment concentration. $0.3\mu\text{M}$ was used in subsequent experiments, as it had an effect on cell growth without causing total cell death by day 5.

We hypothesized that inhibition of Hsp90 would affect the binding of telomeric proteins and chaperones to the telomere. To explore this, H1299 cells were chronically treated for 2, 4 and 8 hours with the Hsp90 inhibitor Radicicol (RAD) at 0.3 μ M (Figure 16A). Quantitation by densitometry of telomere binding by ChIP showed an increase in binding of the telomere proteins TRF1, TRF2, TIN2 and TPP1 after 2 hours of treatment (Figure 16B), suggesting that early inhibition of Hsp90 may allow telomere binding proteins more access to the telomere. It may also suggest that in untreated cells, Hsp90 forms a bridge between the telomeres for stability, which is eliminated with inhibition of Hsp90 causing telomeric proteins to bind the telomere more tightly. This increased binding is reduced by 8 hours, as expected, suggesting a transient effect on telomere binding by Hsp90 inhibition. The increase in binding was statistically significant for TRF2 and TRF1 at 2 and 4 hours, compared to untreated and eight hours, although 2 and 4 hours were not different from each other, nor were 8 hours and untreated ($p < 0.05$). Telomere binding was significantly reduced for Hsp90 at 8 hours compared to untreated and 4 hours ($p < 0.05$) but not 2 hours, while untreated, 2 and 4 hours were not significantly different from each other.

To further explore the effect of Hsp90 inhibition on TRF2's ability to bind the telomere, we hypothesized that TRF2 colocalization with TRF1 after treatment would be transiently disrupted, as was observed with the ChIP data in Figure 16A and B. TRF1 has been shown to be an ideal telomere marker, no matter the treatment conditions (Broccoli, *et al.*, 1997; Karlseder, *et al.*, 2002). Using immunocytochemistry, the TRF2/TRF1 colocalization significantly decreased over time with chronic 0.3 μ M RAD ($p < 0.05$) (Figure 17 and 18) when untreated is compared to all treatment time points, which conflicts with the ChIP data, that shows a significant increase in "telomere binding" at two hours. 4, 8, and 16 hours do not significantly differ from each other or

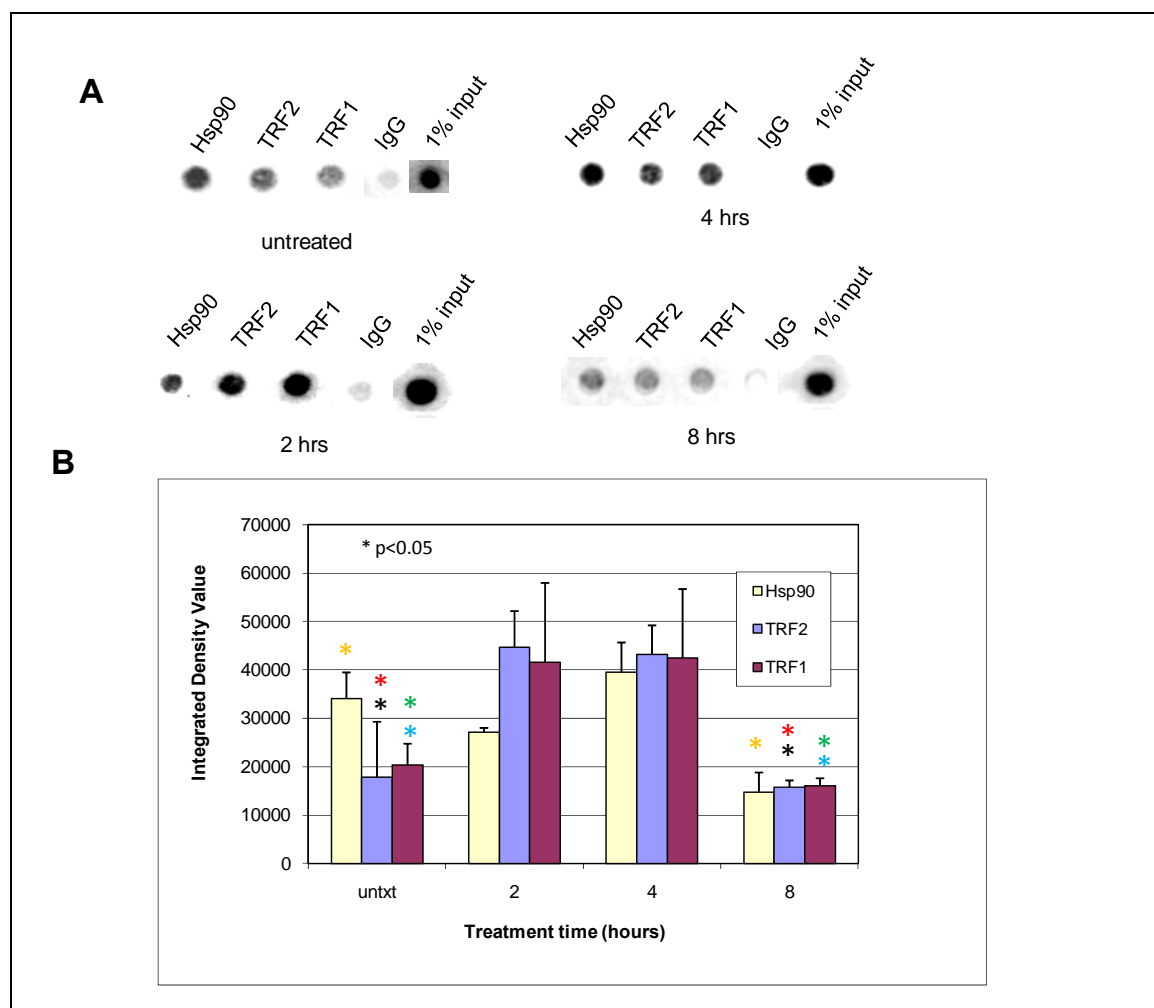


Figure 16. Inhibition of Hsp90 affects TRF2 and TRF1 telomere binding. Using chromatin immunoprecipitation and a telomeric probe, antibodies for the indicated proteins suggest an interaction with telomere sequences after Hsp90 inhibition. **A.** Chronic treatment with 0.3 μ M RAD up to 8 hours was performed on H1299 cells. IgG serves as a negative control. Input is 1-2% sheared DNA and serves as the positive control for hybridization. **B.** Quantitation of telomeric binding with treatment compared to untreated ChIP samples. IgG was subtracted out as background. Duncan's new multiple range test indicates a significant difference ($p < 0.05$) for TRF2 telomere binding at 2 hours compared to 8 hours and untreated (*), and 4 hours compared to 8 hours and untreated (*). For TRF1, Duncan's new multiple range test indicates a significant difference ($p < 0.05$) between 2 hours and 8 hours and 2 hours and untreated (*), 4 hours and 8 hours and 4 hours and untreated (*). Hsp90 telomere binding was significantly different between 8 hours and 4 hours and 8 hours and untreated ($p < 0.05$) (*).

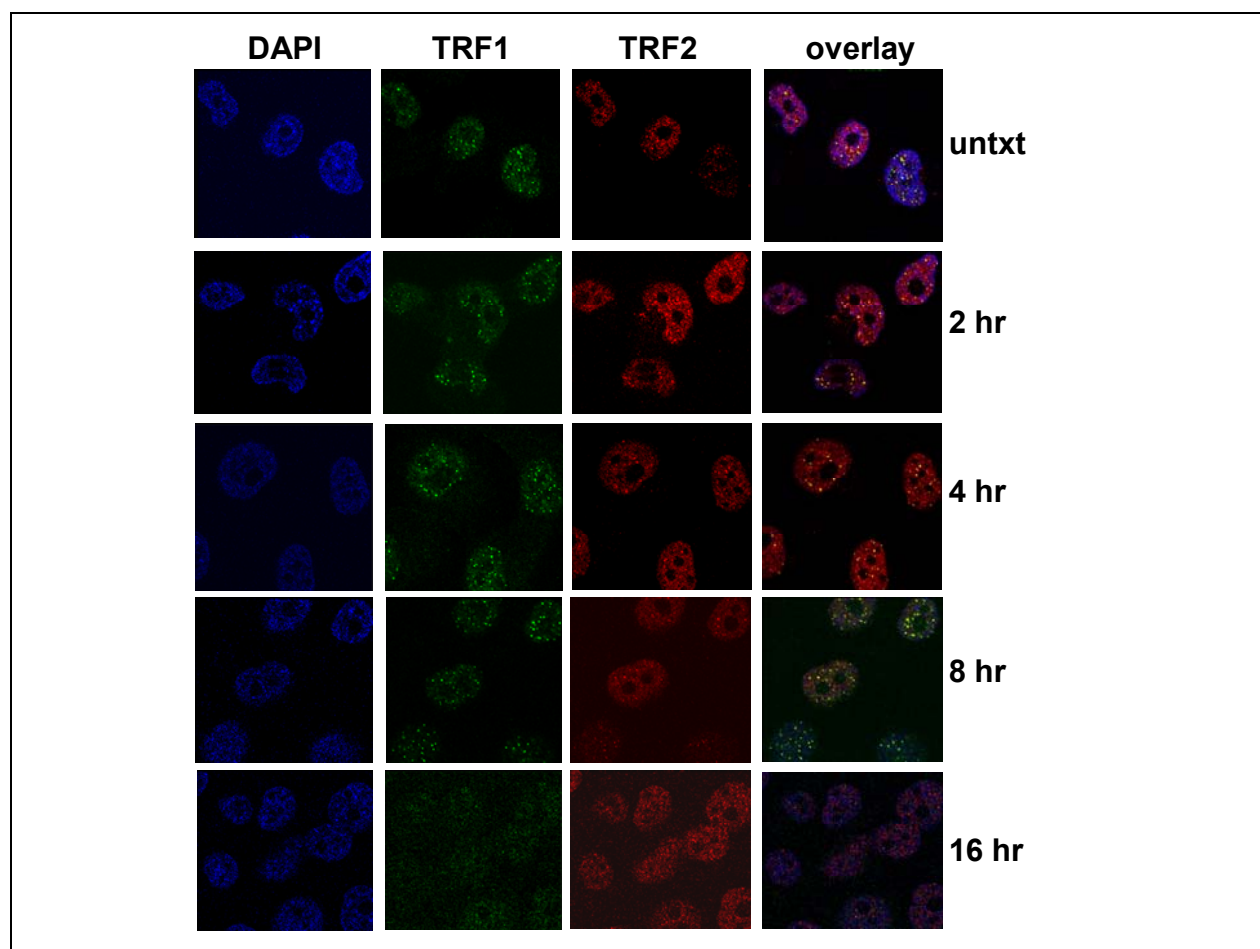


Figure 17. Inhibition of Hsp90 affects TRF1/TRF2 colocalization. H1299 cells were treated for time points indicated with $0.3\mu\text{M}$ Radicicol. Immunocytochemistry shows a transient decrease in TRF2 (Alexa 568) and TRF1 (Alexa 488) interaction with treatment. Nuclear punctate staining of both proteins is retained over time although a reduction in overall TRF1 intensity is observed during treatment. DAPI serves as a control for nuclear staining.

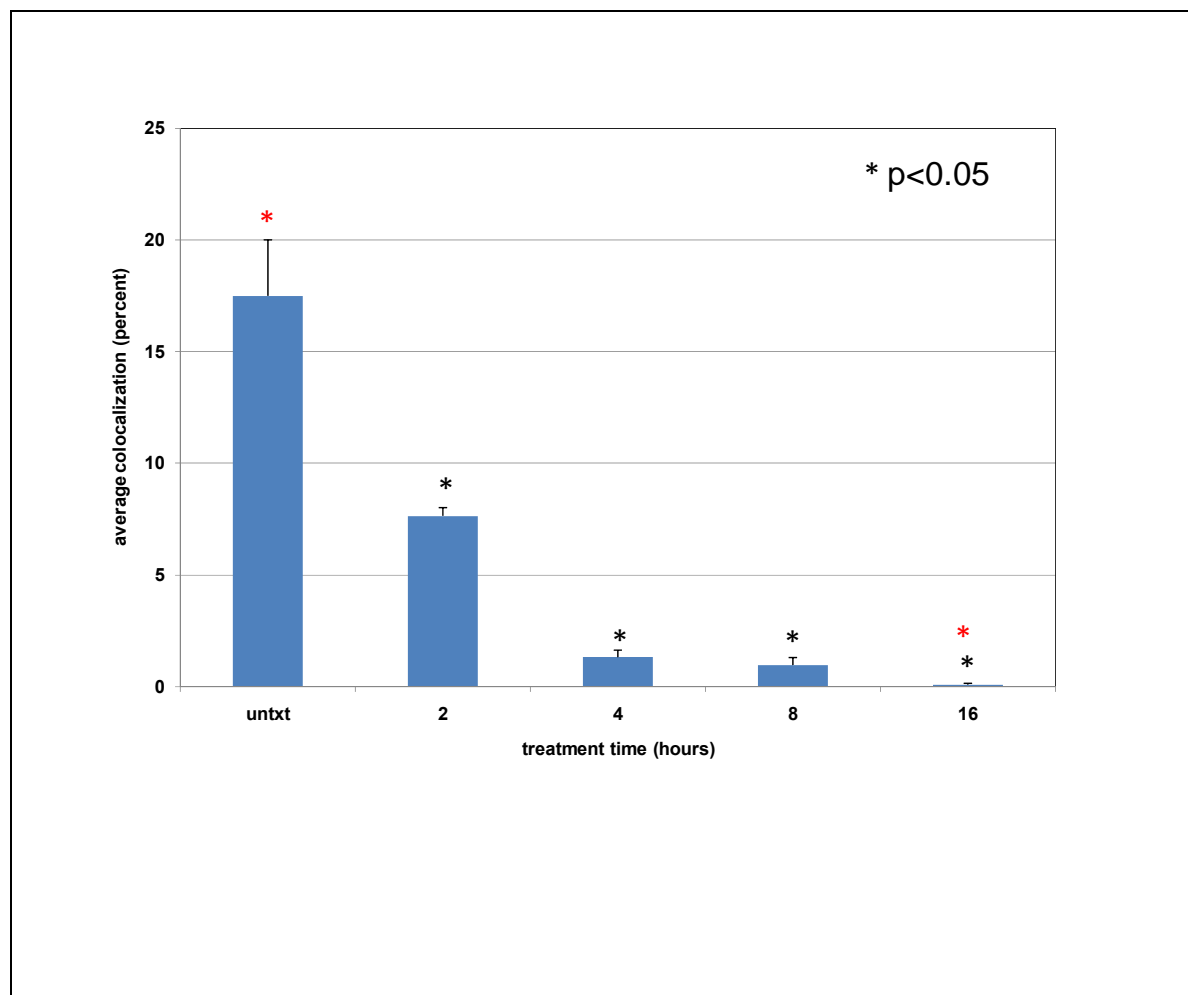


Figure 18. Quantitation of TRF1/TRF2 colocalization. Colocalization analysis using the Leica Lite masked cytofluorogram software average of 3 fields shows nuclear colocalization of TRF1 and TRF2 with chronic 0.3 μ M RAD treatment that decreases over time. A Duncan's new multiple range test indicates a significant difference ($p < 0.05$) for untreated when compared to all time points (*). 2 hours is significantly different from 16 hours and untreated (*).

from 2 hours, while 2 hours is significantly different from 16 hours ($p < 0.05$). The results in Figures 16, 17, and 18 suggest that Hsp90 plays some role in TRF1 and/or TRF2's ability to bind to the telomere, where absence of Hsp90 at the telomere results in a transient increase followed by a reduction of telomeric protein binding over time.

To examine the effect Hsp90 inhibition has on the interaction between TRF1/TRF2 and Hsp90/Hsp70 using the H1299 cell line, cells were chronically treated with 0.3 μ M Radicicol for 2, 4, 8, and 16 hours. We hypothesized that the reduction in telomere binding visualized by ChIP after RAD treatment would be reflected in a reduction in Hsp90, TRF2 and TRF1 protein levels and in Hsp90/TRF2 and Hsp90/TRF1 interactions. Sonicated whole cell lysates were obtained and Western blots were probed for Hsp90, Hsp70, and TRF2. As shown in Figure 19A and B, a trend toward decreased Hsp90 and Hsp70 levels was observed at 2 hours up to 8 hours, with an increase at 16 hours. TRF2 level fluctuations were minimal, while p23 levels seemed relatively unaffected. Quantitation of protein levels by densitometry suggest this trend in fluctuation of Hsp90 and Hsp70 levels with chronic RAD treatment was not significant. Long term chronic treatment with 0.3 μ M RAD up to five days from one independent experiment showed no change in Hsp90 or Hsp70 levels (Figure 19C and 19D), suggesting RAD's effect on proteins is early and transient.

To further determine the effect RAD exerts on Hsp90, H1299 cells were treated with 0.3 μ M RAD for 2 hours. Hsp90, normally localized primarily to the cytoplasm (Figure 20, left panel), can be seen throughout the cell after treatment, including in the nucleus (as identified by the DAPI staining) (Figure 20, right panel). The change in localization of Hsp90 suggests that after inhibition, Hsp90 relocates to the nucleus to act on nuclear proteins, such as TRF1 and TRF2 in response to RAD treatment.

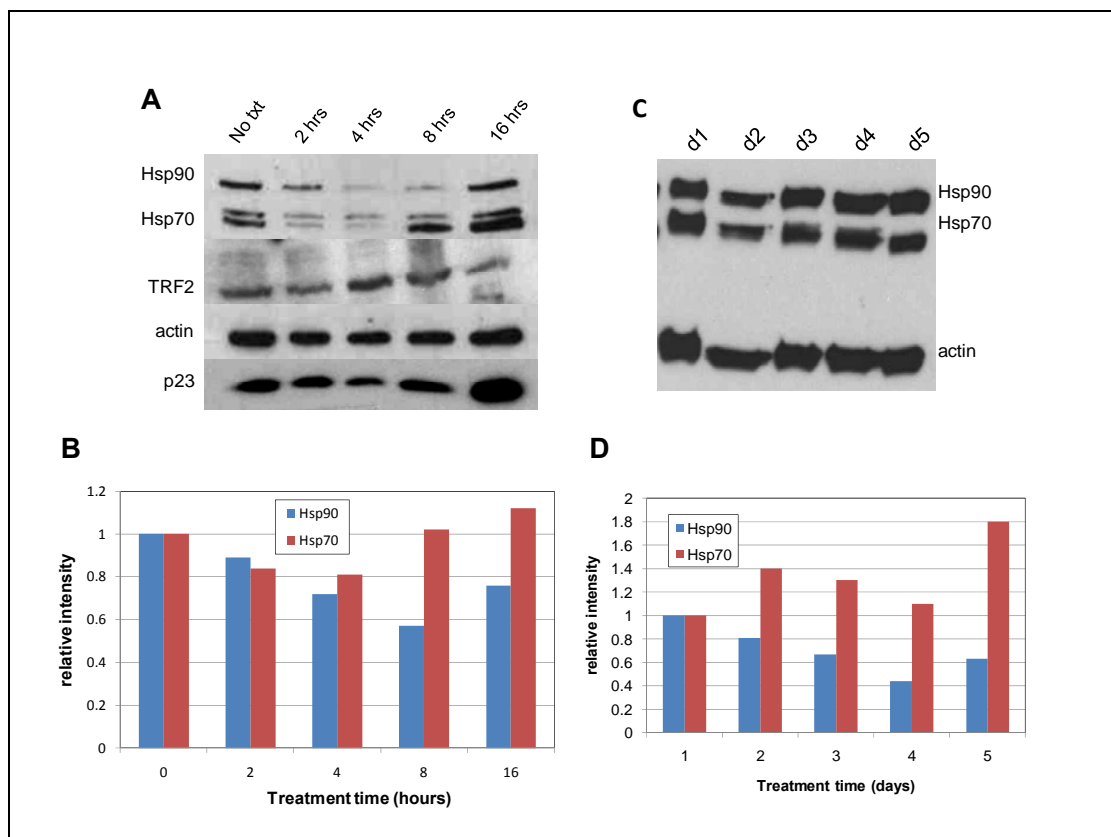


Figure 19. Hsp90 inhibition results in variable change in chaperone protein levels but no change in TRF2 protein levels in H1299 cells. **A.** A Western blot of 50 μ g protein (for Hsp90, Hsp70, Actin and p23) or 100 μ g of protein (for TRF2) from sonicated whole cell extracts was performed after treatment with 0.3 μ M Radicicol for indicated time points. Actin serves as a loading control. **B.** Quantitation of protein level change in part A by densitometry from two separate experiments. Cells were first normalized to the loading control and then normalized to untreated controls (IDV set to a value of 1). **C.** Cells were chronically treated for 5 days with 0.3 μ M Radicicol. Western blot probed for Hsp90 and Hsp70 is shown (50 μ g protein loaded). Actin serves as a loading control. **D.** Quantitation of protein levels in part C by densitometry from one experiment. Cells were first normalized to the loading control and then normalized to day 1 (IDV set to a value of 1).

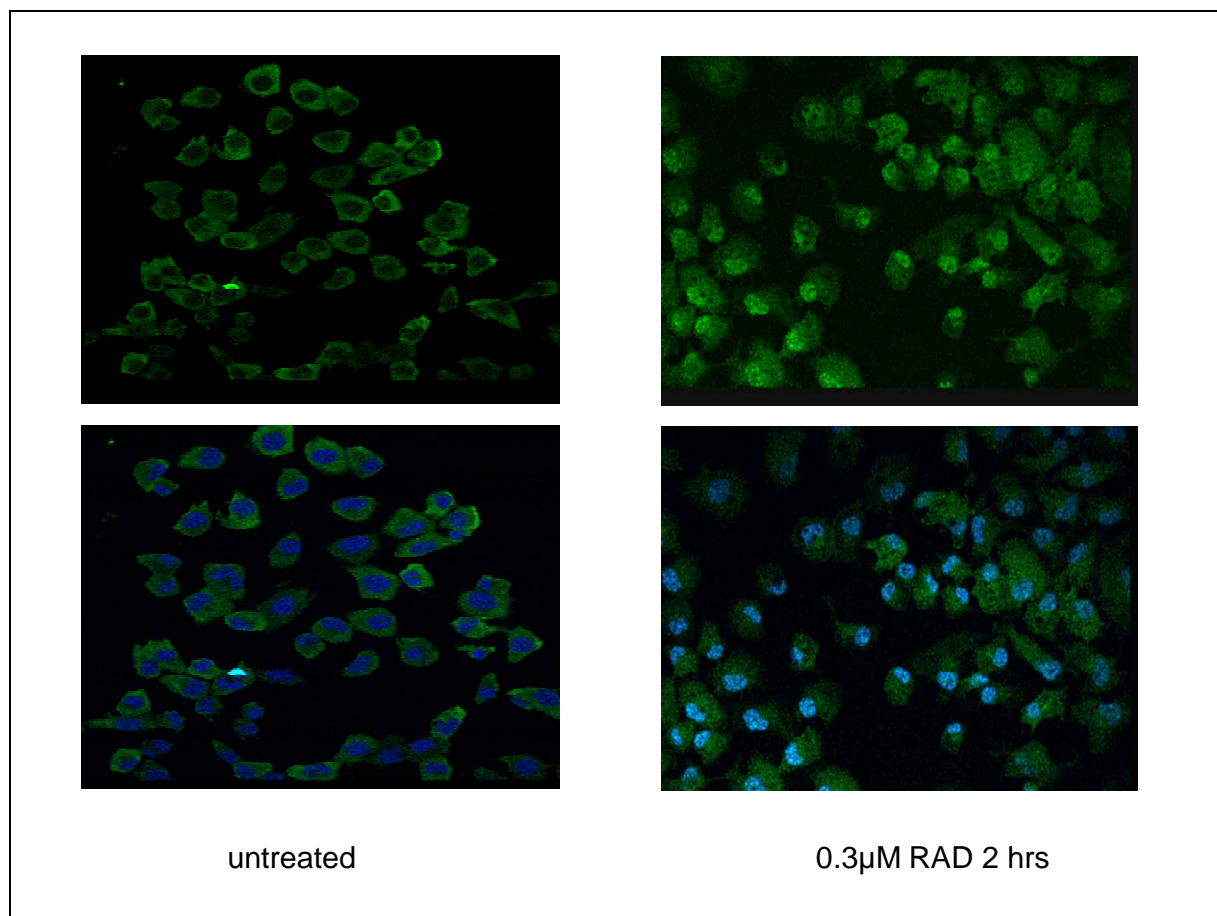


Figure 20. Inhibition of Hsp90 disrupts Hsp90 localization in H1299 cells. After chronic treatment with 0.3 μ M Radicicol for two hours, Hsp90 shows a marked change in localization from primarily cytoplasmic (left panel, untreated) to cytoplasmic and nuclear (right panel). DAPI indicates nuclear staining (bottom panels).

Inhibition of Hsp90 affected its localization in the cell as well as TRF1 and TRF2's ability to bind to the telomere. We observed that the interaction between Hsp90/Hsp70 and TRF2/TRF1 was also affected by inhibition of Hsp90. Up to 16 hours of chronic treatment with 0.3 μ M RAD resulted in a disruption of the interaction between TRF2 and chaperones, as evidenced by Co-IP/Western of nuclear extracts. Only the 16 hour Hsp70/TRF2 interaction was significantly disrupted when compared to all time points and untreated ($p < 0.05$) (Figure 21A and 21B) and at 8 hours the TRF2/Hsp90 interaction was significantly disrupted when compared to untreated (Figure 21B). To ensure that the timing of the sample collection had no effect on the interaction, samples were all collected at the same end point, 16 hours (with treatment for the amount of time indicated) (Figure 21A, bottom panel), while in Figure 21A, top panel, samples were all treated at the same start time, followed by collection at the time points indicated. Results in the two experiments were nearly identical, suggesting that experimental protocol had no effect on the disruption of the chaperone/TRF2 interactions. Cytoplasmic extracts Co-IP'd for TRF2 and probed for Hsp90 and Hsp70 showed no interaction between Hsp90, Hsp70 and TRF2 in untreated cells as would be expected because TRF2 is a nuclear protein and Hsp90 localizes to the nucleus after treatment (Figure 21C, Figure 20). An interaction between Hsp70 and TRF2 is observed after RAD treatment, which peaked at 8 hours, while the Hsp90/TRF2 interaction remained almost undetectable even after treatment (Figure 21C). The presence of a modest Hsp90/TRF2 interaction at 8 hours (Figure 21C and 21D) suggests that (a very small fraction of) TRF2 may be exported to the cytoplasm for proteasome-mediated degradation mediated by Hsp90. The strong interaction of Hsp70 and TRF2 in the cytoplasm was unexpected. Given this unusual finding, it is possible that the Hsp70/TRF2 interaction is an artifact of the Co-IP technique as we find little data to support the cytoplasmic localization of TRF2.

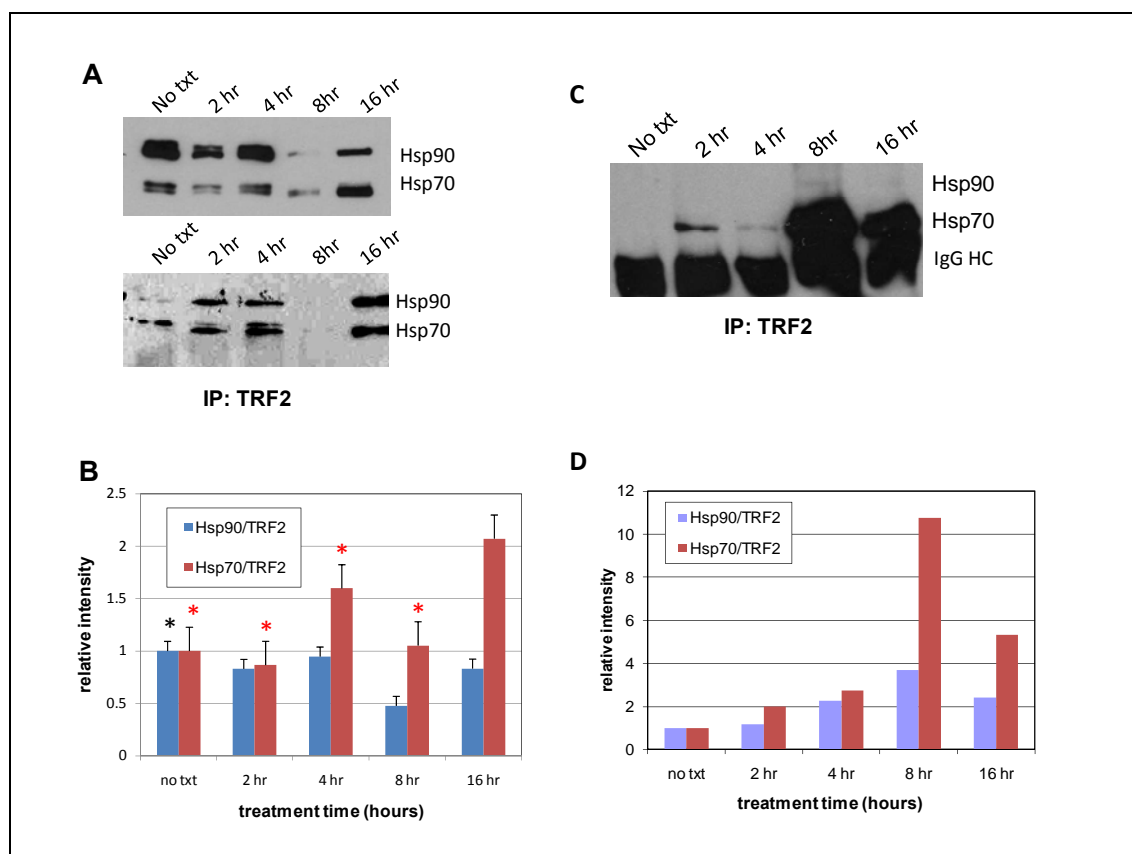


Figure 21. Hsp90 inhibition affects interaction levels between chaperones and TRF2 in nuclear and cytoplasmic extracts of H1299 cells. **A.** Cells were treated with 0.3 μ M Radicicol for 2, 4, 8, and 16 hours. Nuclear extracts were then immunoprecipitated with TRF2 antibody and Western blots for Hsp90 and Hsp70 were performed. In the top panel, samples were treated together and collected at time points indicated. In the bottom panel, treatment time points were staggered so that the collection occurred at the 16 hr time point. **B.** Quantitation through densitometry of five separate experiments and Duncan's new multiple range test shows a significant change in the interaction between TRF2 and Hsp90 at 8 hours compared to untreated samples ($p < 0.05$) (*) and between Hsp70/TRF2 at 16 hours compared to all other treatments (*). Samples were normalized to untreated samples, set to an IDV value of 1. **C.** Cells were treated with 0.3 μ M Radicicol for 2, 4, 8, and 16 hours. Cytoplasmic extracts were then immunoprecipitated with TRF2 antibody and Western blots were probed for Hsp90 and Hsp70. **D.** Quantitation through densitometry of a single cytoplasmic experiment from (C). Samples were normalized to untreated samples, set to an IDV value of 1.

The interaction between Hsp90, Hsp70 and TRF1 was also disrupted by inhibition of Hsp90 in H1299 cells, although the disruption differs from that seen with TRF2/Hsp90. At 8 hours, there is a significant increase ($p<0.05$) in the Hsp90/TRF1 interaction compared to untreated and to 2, 4, and 16 hour time points as evidenced by Co-IP/Western of nuclear extracts (Figure 22A and 22B). The Hsp70/TRF1 interaction at 16 hours is significantly different from 2 hours and untreated, while 8 hours is significantly different from 2 hours, 4 hours, and untreated samples ($p<0.05$) (Figure 22A and 22B). Compared to TRF2 where we observed a reduction that is significant at 8 hours (Figure 21B), we observed a significant increase in the TRF1/Hsp90 interaction at 8 hours (Figure 22B). The difference in timing between the Hsp90 interactions with TRF1 and TRF2 suggests that Hsp90 may be binding to one protein in response to treatment to stabilize it and then switching to the other, although this has yet to be determined.

To confirm results from the Co-IP/Westerns, we examined Hsp90/TRF2 interaction in cells using immunocytochemistry. TRF2, as a nuclear telomeric protein, normally displays nuclear punctate immunofluorescent staining in cells. We chronically treated cells up to 16 hours with 0.3 μ M RAD and assessed changes in colocalization with Hsp90 and punctate staining of TRF2. Using the Leica LCS Lite image analysis software, pixel distribution was analyzed for colocalization of two fluorophores at once. The masked cytofluorogram program quantifies and analyses the percentage of colocalization between Alexa 488 (Hsp90) and Alexa 568 (TRF2) for nuclear colocalization of pixels from three separate fields. No differences in TRF2 punctate staining were observed over time, and Hsp90 and TRF2 colocalized to varying degrees at all time points (Figure 23 and 24). Quantitation of colocalization using the Leica Lite masked cytofluorogram software revealed that nuclear colocalization of Hsp90 and TRF2 significantly increased at 4 hours ($p<0.05$) above the untreated and all other time points (Figure 24). 16 hour

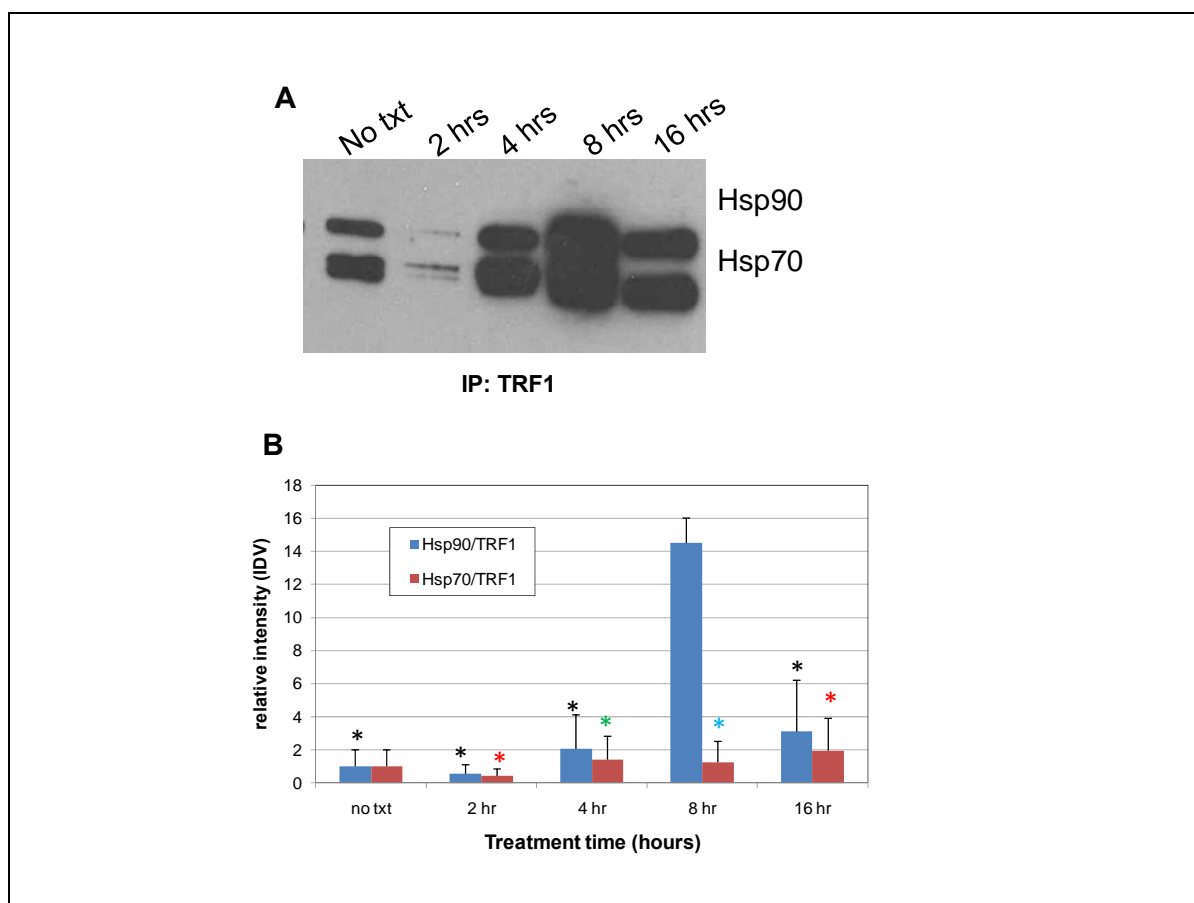


Figure 22. Hsp90 inhibition affects interaction levels between chaperones and TRF1 in H1299 cells. **A.** Cells were treated with 0.3 μ M Radicicol for 2, 4, 8, and 16 hours. Nuclear extracts were then immunoprecipitated with TRF1 antibody and Western blots for Hsp90 and Hsp70 were performed. **B.** Quantitation through densitometry of three separate experiments shows a significant difference (Duncan's new multiple range test, $p < 0.05$) in the interaction between TRF1 and Hsp90 with 8 hours compared to all treatment times (*). The Hsp70/TRF1 indicates a significant difference between 16 hours, 2 hours and untreated (*) and between 8 hours and 2 hours (*), 4 hours and untreated (*) ($p < 0.05$). Samples were normalized to untreated samples, which was set to an IDV value of 1.

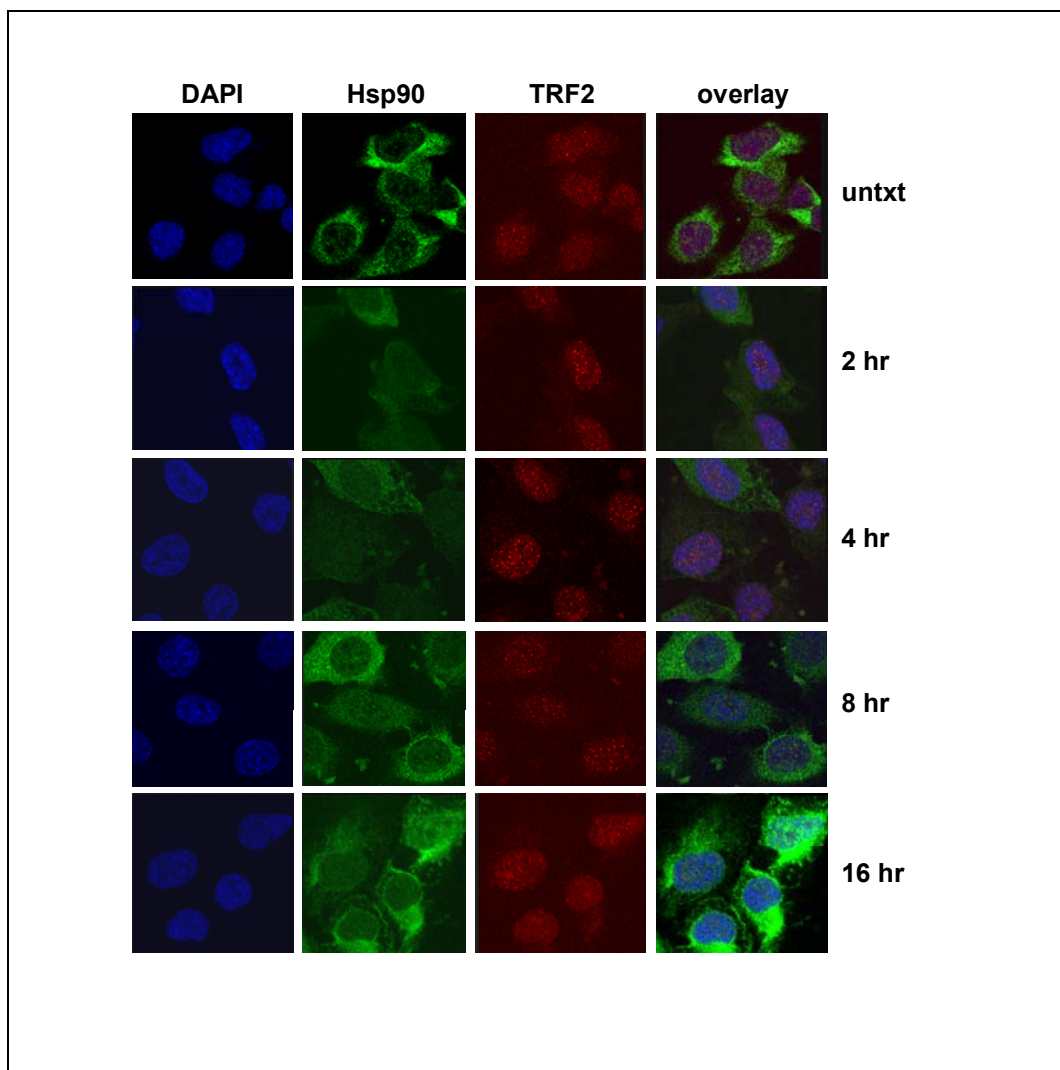


Figure 23. Inhibition of Hsp90 affects the colocalization of Hsp90 and TRF2 in H1299 cells. H1299 cells were chronically treated for time points indicated with $0.3\mu\text{M}$ Radicicol. Immunocytochemistry shows no change in TRF2 (Alexa 568) in the nucleus with treatment as punctate staining is retained over time. Hsp90 (Alexa 488) primarily stains the cytoplasm, with a change in localization beginning at 2 hours of treatment up to 16 hours, where it can be seen in the nucleus colocalizing with TRF2. DAPI serves as a control for nuclear staining.

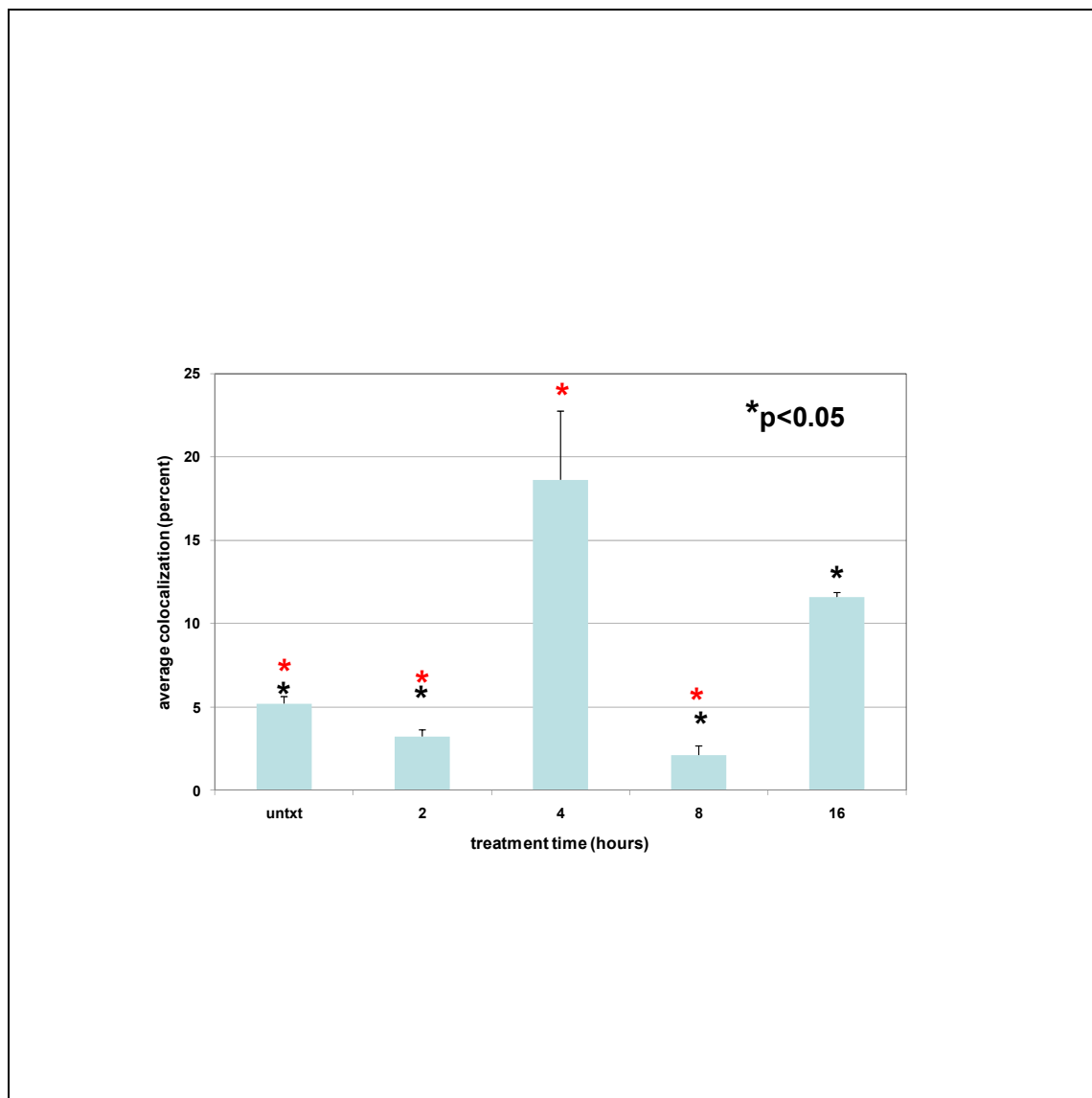


Figure 24. Quantitation of TRF2/Hsp90 colocalization. Colocalization analysis using the Leica Lite masked cytofluorogram software average of 3 fields shows a transient change in nuclear colocalization of Hsp90 and TRF2 with RAD treatment over time. Duncan's new multiple range test suggests a significant change at 4 hours (*) and 16 hours (*) compared to all time points and untreated ($p < 0.05$).

was also significantly different from all time points and untreated ($p < 0.05$) (Figure 24). The transient change in the nuclear colocalization between Hsp90 and TRF2 almost perfectly parallels the transient changes observed in Co-IP/Western results (Figure 21A). No detectable changes in TRF2 localization suggest that Hsp90 inhibition has no deleterious effect on the TRF2 protein itself (i.e. degradation) (Figure 23), as protein levels are relatively unaffected with treatment (Figure 19A). In addition, RAD treatment does not appear to induce exportation to the cytoplasm for proteasome-mediated degradation. Colocalization signals seen in the cytoplasm are likely due to background or small amounts of TRF2 localizing to the cytoplasm for degradation due to normal protein turnover (Figure 23), as we previously showed with a lack of Hsp90 and TRF2 interaction in the cytoplasm (Figure 21C).

TRF1, as a telomeric protein, also normally displays nuclear punctate immunofluorescent staining in cells. We chronically treated cells up to 16 hours with $0.3\mu\text{M}$ RAD and assessed changes in colocalization with Hsp90 and punctate staining of TRF1. TRF1 localization remained nuclear and mostly punctate with RAD treatment (Figure 25), although TRF1 appeared more diffuse at 2 and 4 hours. Quantitation by the Leica Lite software showed that nuclear colocalization of Hsp90 and TRF1 significantly increased at 8 and 16 hours compared to untreated and 2 and 4 hours ($p < 0.05$) (Figure 26) similar to results from the Co-IP/Westerns, which showed a significant increase in Hsp90/TRF1 nuclear interaction at 8 hours and almost no interaction at 2 hours (Figure 22A). Again, this may be indicative of an Hsp90 protein interaction switching from TRF2 to TRF1 due to RAD treatment. Untreated, 2 hours and 4 hours were not significantly different from each other nor were 8 and 16 hours.

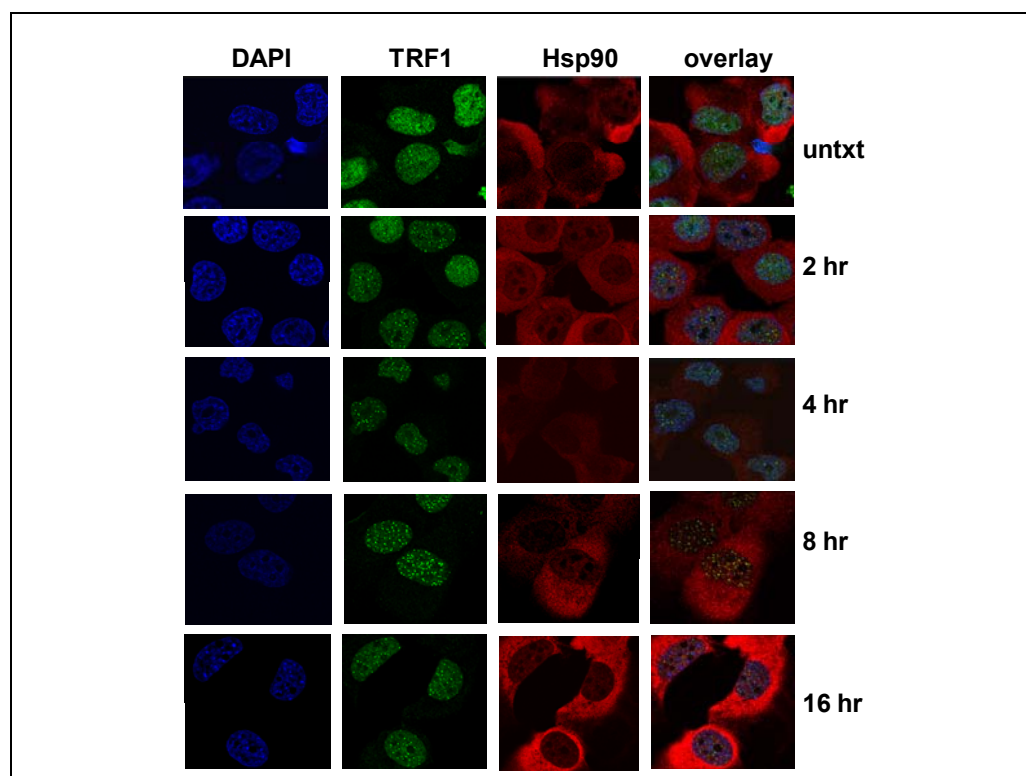


Figure 25. Inhibition of Hsp90 transiently affects the colocalization of Hsp90 and TRF1 in H1299 cells. H1299 cells were treated for time points indicated with 0.3 μ M Radicicol. Immunocytochemistry shows nuclear TRF1 (Alexa 488), while Hsp90 (Alexa 568) stains the cytoplasm and nucleus. A transient change in Hsp90/TRF1 colocalization was observed. DAPI serves as a control for nuclear staining.

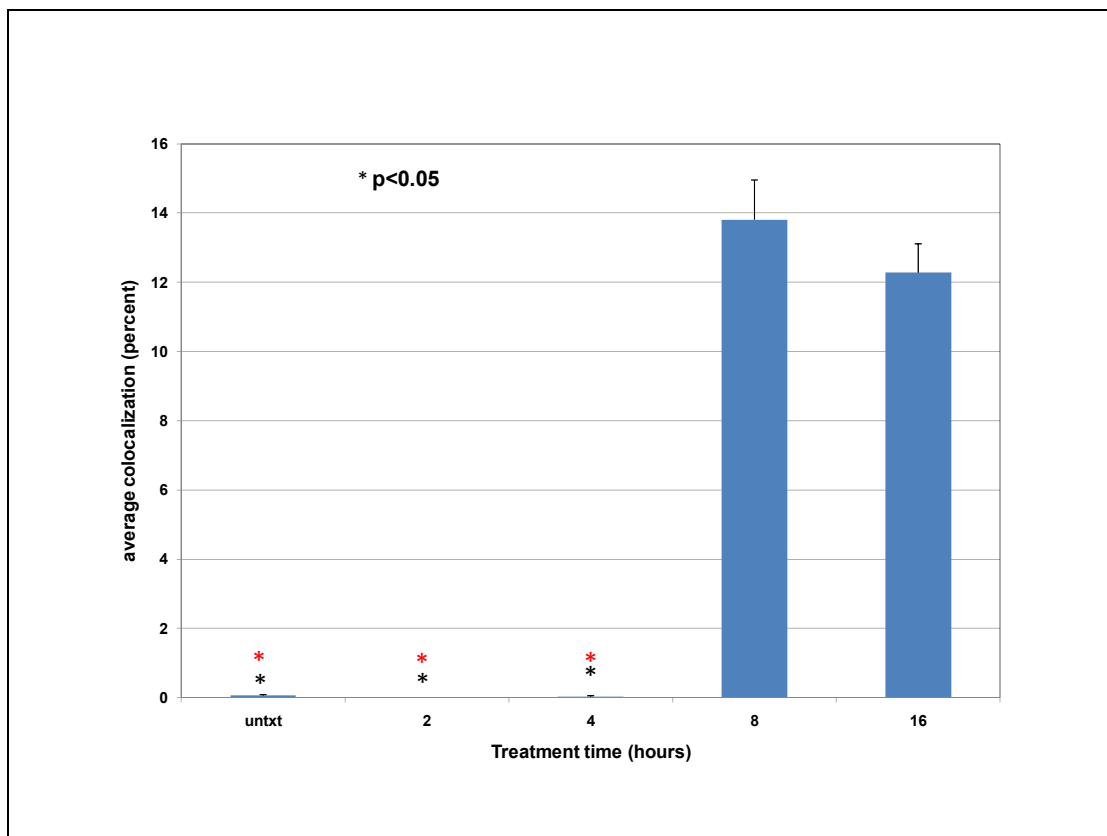


Figure 26. Quantitation of TRF1/Hsp90 colocalization. Analysis by Leica Lite masked cytofluorogram colocalization software of an average of 3 fields showed nuclear co-localization of TRF1 and Hsp90 increased significantly at 8 (*) and 16 (*) hours compared to untreated, 2 and 4 hours (Duncan's new multiple range test, $p < 0.05$).

TRF2, TRF1 and POT1 are the only Shelterin proteins that bind directly to telomeric DNA, but a weak signal from TIN2 and TPP1 may be seen if they are closely interacting with TRF2 or TRF1, as evidenced by ChIP data (Figure 10A). One function of Hsp90 is to aid in protein-protein interactions of its client proteins. Because we observed that Hsp90 also interacts with the telomeric proteins TIN2 and TPP1, and Hsp90 inhibition effects TRF1/TRF2 interaction (Figure 12D, 17 and 18), we hypothesized that the interaction of Hsp90 with TRF2 may function in its interactions with other telomere proteins such as TIN2 and TPP1, possibly enhancing or preventing direct interactions. To explore this, we inhibited Hsp90 using chronic RAD treatment at 0.3 μ M up to 6 hours. Immunocytochemistry revealed a significant increase in TRF2/TIN2 colocalization with chronic treatment of 0.3 μ M RAD at 2 hours ($p < 0.05$) (Figure 27 and Figure 28). A significant increase in TRF2/TPP1 colocalization with treatment was observed at all time points ($p < 0.05$) compared to untreated, as well as at 1 hour compared to 2 hours treatment ($p < 0.05$) (Figure 29 and Figure 30). Together, these data suggest that inhibition of Hsp90 effects TIN2 and particularly TPP1's ability to interact with TRF2 compared to untreated samples. This is especially interesting for the TRF2/TPP1 interaction, as these proteins do not normally interact directly but associate through the TRF2/TIN2 interaction. ChIP data showed an increase in TIN2 and TPP1's ability to bind to the telomere after 2 hours of chronic RAD treatment (data not shown), suggesting an increase in telomere binding of TIN2 and TPP1 (or association with telomere binding proteins such as TRF1 and TRF2) as was reflected in the immunocytochemistry results shown in Figure 27-30. The apparent increase in telomeric access is reflected in the increase in TRF2 colocalization with both TIN2 and TPP1, suggesting that Hsp90 has a direct effect on TRF2's ability to bind other Shelterin proteins. Therefore, we have determined that Hsp90 interacts with TRF1, TRF2, TIN2 and TPP1 and that inhibition of Hsp90

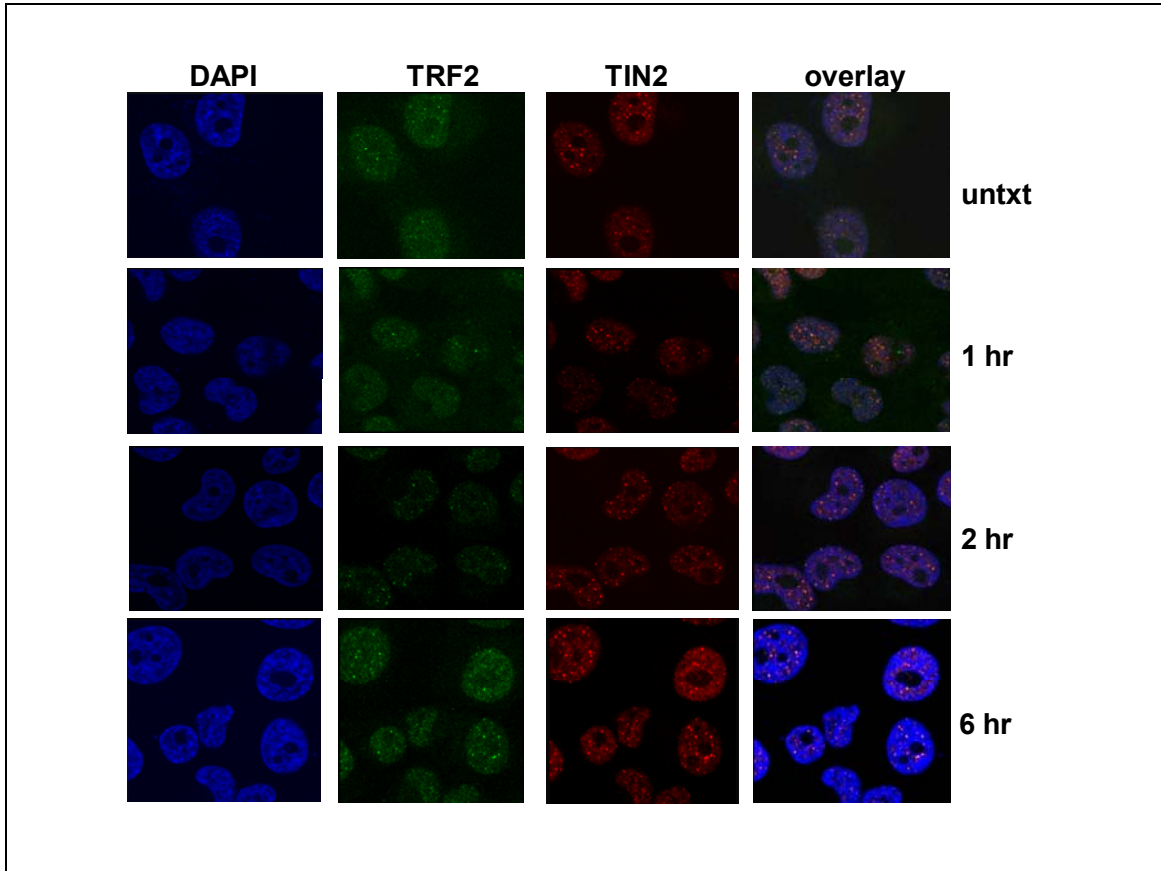


Figure 27. Inhibition of Hsp90 transiently affects the colocalization of TRF2 and TIN2 in cells. H1299 cells were chronically treated for time points indicated with 0.3 μ M Radicicol. Immunocytochemistry shows an increase in TRF2 (Alexa 488) colocalization with the telomere associated protein TIN2 (Alexa 568) at 2 hrs. DAPI serves as a control for nuclear staining.

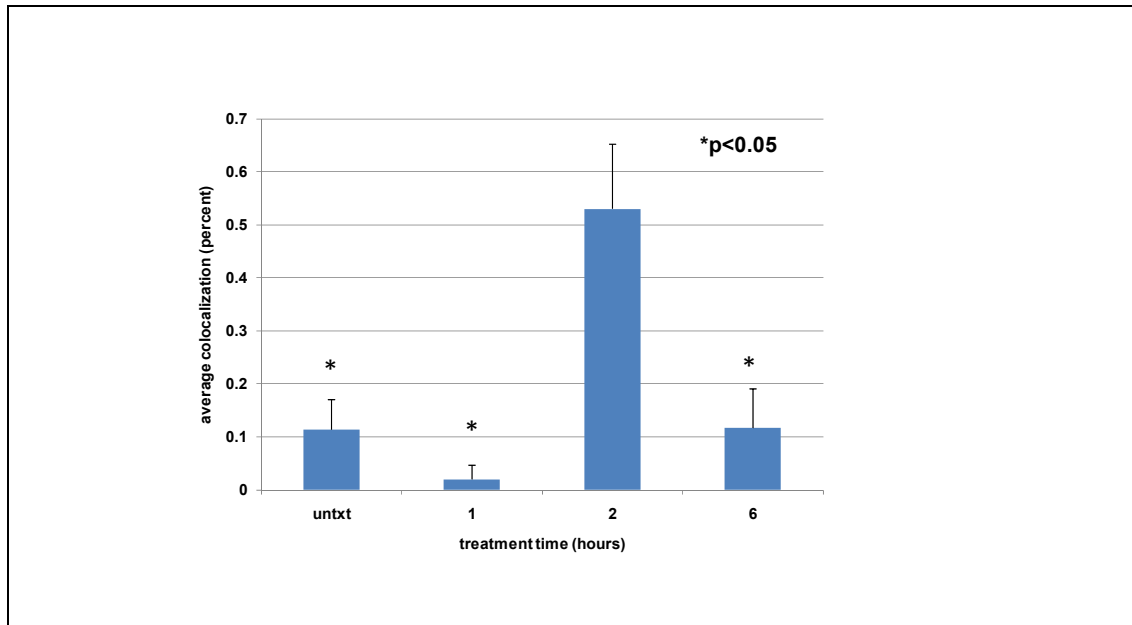


Figure 28. Quantitation of TRF2/TIN2 colocalization. Analysis by Leica Lite masked cytofluorogram colocalization software of 3 fields showed nuclear co-localization of TRF2 and TIN2 increased significantly at 2 hours compared to all other time points (Duncan's new multiple range test (*)) ($p < 0.05$).

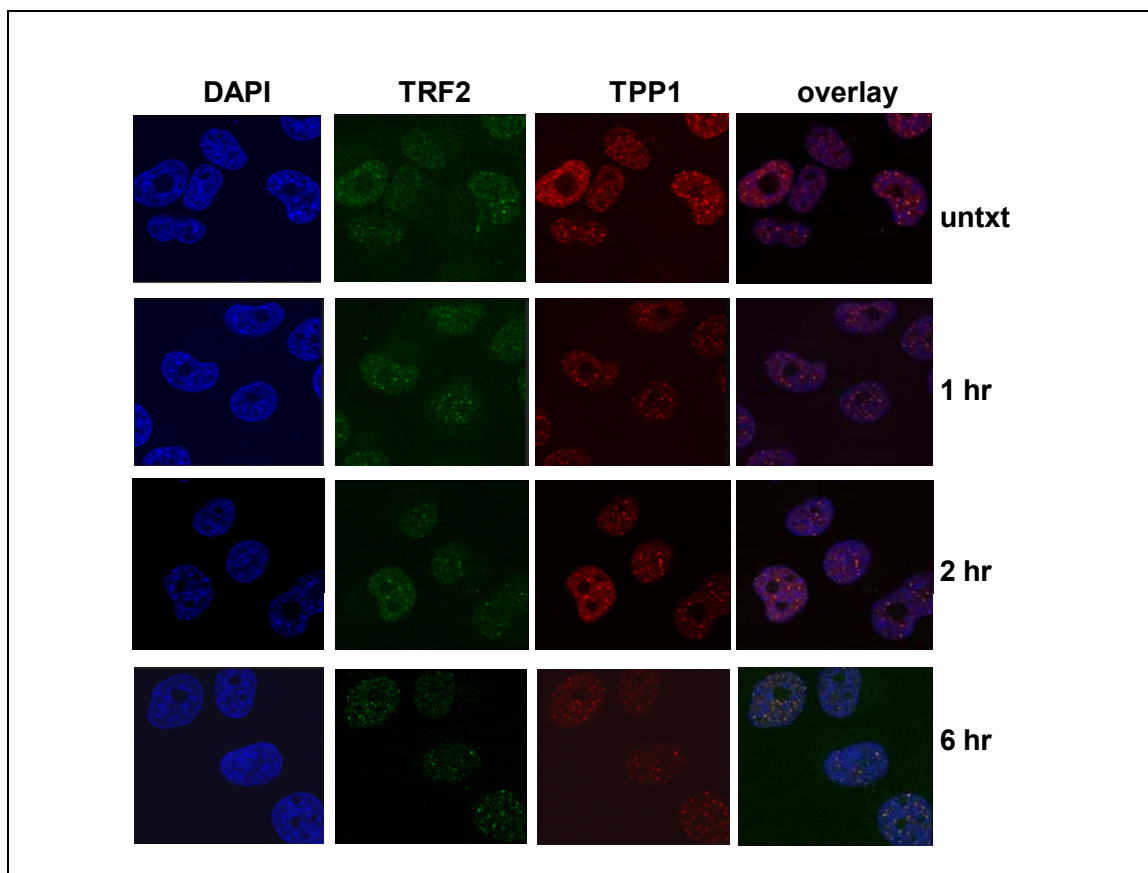


Figure 29. Inhibition of Hsp90 has an effect on the colocalization of TRF2 and TPP1 in cells. H1299 cells were treated for time points indicated with 0.3 μ M Radicicol. Immunocytochemistry shows an increase in TRF2 (Alexa 488) colocalization with the telomere associated protein TPP1 (Alexa 568) at each time point. DAPI serves as a control for nuclear staining.

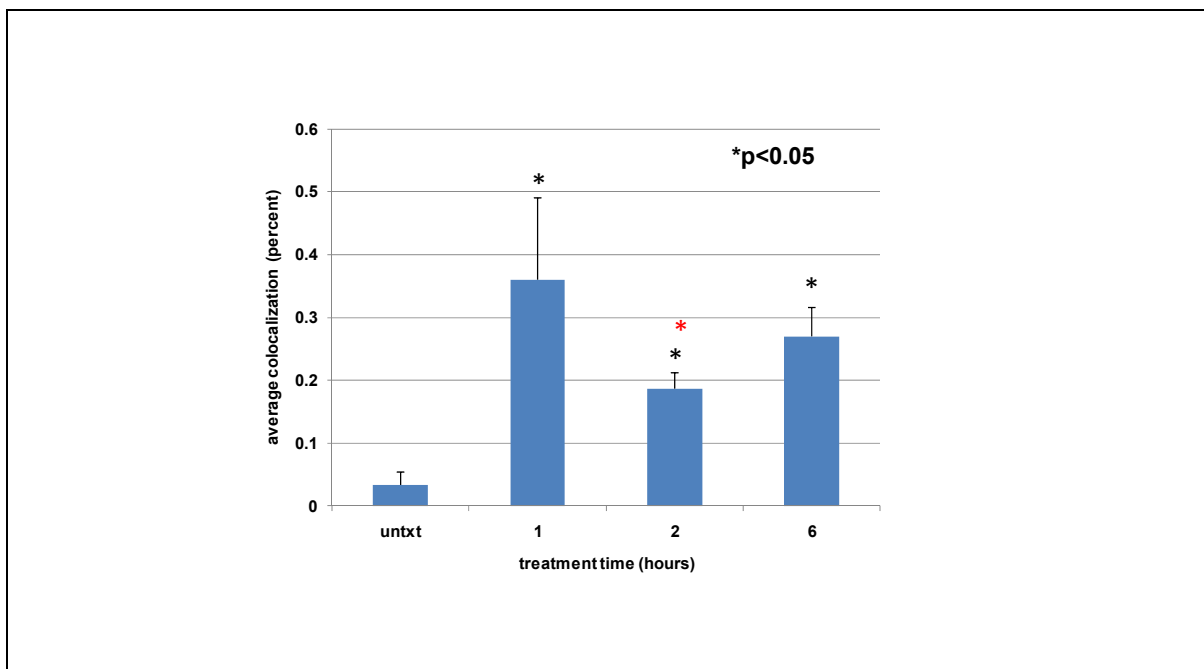


Figure 30. Quantitation of TRF2/TPP1 colocalization. Analysis of nuclear colocalization by the Leica Lite masked cytofluorogram shows a transient change in colocalization of TRF2 and TPP1 over time, which is significantly increased at all time points compared to untreated controls (Duncan's new multiple range test (*) ($p < 0.05$)). 1 hour is significantly increased compared to 2 hours (*) ($p < 0.05$).

affects TRF2's ability to colocalize with TRF1, TIN2 and TPP1. Inhibition of Hsp90 also affects Hsp90's ability to interact with TRF1 and TRF2, all suggesting a role for Hsp90 in protein-protein interactions at the telomere.

Ubiquitination of TRF2

Inhibition of Hsp90 is known to target client proteins for proteasome-mediated degradation (Shulte, *et al.*, 1997). We hypothesized that through protein-protein interactions and telomere binding, Hsp90 may be stabilizing TRF2 and/or TRF1 at the telomere in cancer cells; therefore, inhibition of Hsp90 could result in degradation of TRF2 and/or TRF1. An initial experiment (Figure 21C) suggests that TRF2 is not interacting with Hsp90 in the cytoplasm, yet it remains possible that TRF2 is being degraded quickly and we are unable to account for it in the Co-IP/Westerns. To determine if TRF2 was interacting with chaperones in the cytoplasm, thus suggesting proteasome-mediated degradation, cells were chronically treated with 0.3 μ M RAD and 10 μ M of the proteasome inhibitor MG132. If Hsp90 targets TRF2 for proteasome-mediated degradation, MG132 could prevent degradation of TRF2 and cause localization to the cytoplasm, as it would still be exported from the nucleus but not degraded. Nuclear and cytoplasmic extracts were Co-IP'd after treatment for TRF2 followed by Western analysis for Hsp90 and Hsp70. Nuclear extracts showed disruption in interactions between Hsp90 and TRF2 at 2 and 4 hours (Figure 31, left panel), which differed slightly from that seen in Figure 21A. It is important to note that Hsp90 and TRF2 still interact over time and addition of MG132 does not seem to have an effect on the Hsp90/TRF2 association, suggesting that Hsp90 inhibition does not cause TRF2 degradation. In cytoplasmic extracts, Hsp90/TRF2 interactions are undetectable (Figure 31, right

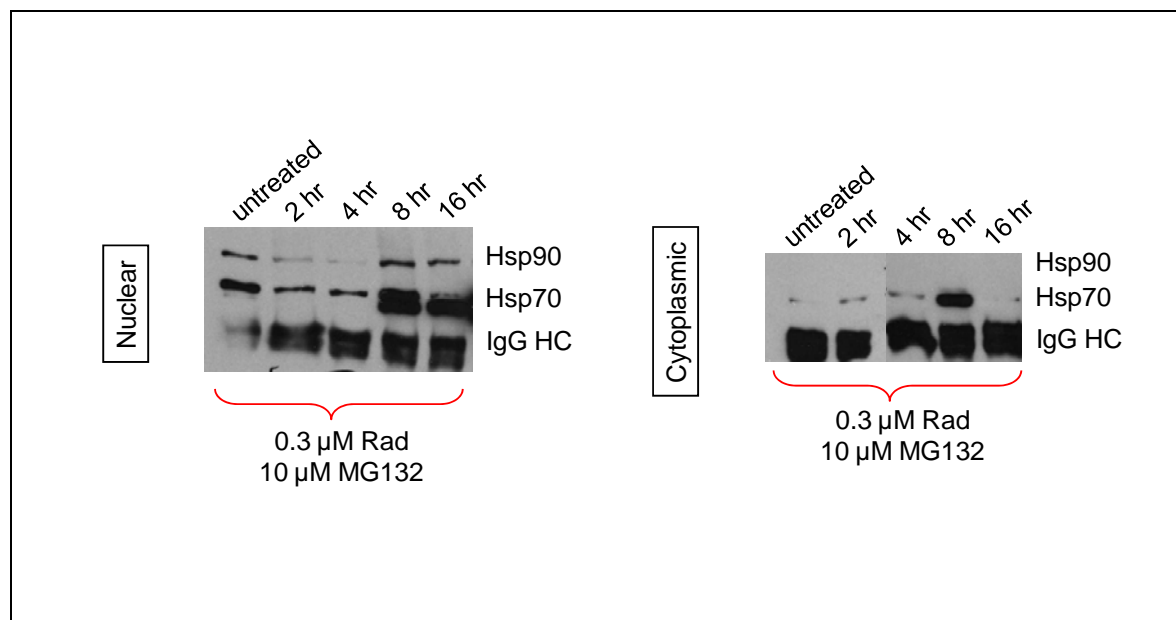


Figure 31. Hsp90 inhibition has little effect on chaperone and TRF2 interaction and localization. H1299 cells were treated with 0.3μM Radicicol with the proteasome inhibitor MG132 (10μM) at the times indicated. Nuclear and cytoplasmic extracts were immunoprecipitated for TRF2 and Western blots were probed for Hsp90 and Hsp70. IgG serves as a negative control. Hsp90 and TRF2 only interact in the nucleus, while Hsp70 and TRF2 interact in both the nucleus and the cytoplasm.

panel), as observed previously (Figure 21C). Consistent with Figure 21C, the Hsp70/TRF2 interaction was observed in nuclear and cytoplasmic extracts after treatment, suggesting again the potential for artifactual IP results as there is no evidence for TRF2 exportation to the cytoplasm (Figure 21C and Figure 31, right panel).

To verify the results and ensure that TRF2 is not interacting with Hsp90 as a means to be targeted for degradation, further examination of TRF2 ubiquitination was performed. H1299 cells were chronically treated with 0.3 μ M RAD with or without 10 μ M MG132 up to 24 hours. Co-IPs were performed for TRF2 and Westerns were probed for Ubiquitin. Ubiquitination of TRF2 displays a characteristic laddering signal above TRF2. When nuclear and cytoplasmic extracts were compared, more ubiquitination was observed in the cytoplasm in Figure 32 (lower panel), but this low intensity pattern likely was the result of normal TRF2 protein turnover rather than a drug-induced degradation. In addition, low intensity bands in the nuclear extracts were likely also due to normal protein turnover (Figure 32, upper panel). Collectively, our data suggests that Hsp90 inhibition by Radicicol treatment does not cause TRF2 degradation.

Our results show that when H1299 cells were chronically treated with 0.3 μ M RAD and 10 μ M MG132 for 2 hours TRF1 appeared to be ubiquitinated in response to Hsp90 inhibition (Figure 33), suggesting Hsp90 inhibition results in targeting TRF1 for degradation. In an effort to determine if TRF1 is ubiquitinated, nuclear extracts were collected and immunoprecipitated against TRF2, TRF1, IgG, and Ubiquitin in treated and untreated samples and Western blots were probed for Ubiquitin. Consistent with previous results (Figure 21C and Figure 32), the levels of TRF2 ubiquitination did not differ from basal levels of degradation and can be explained by normal protein turnover.

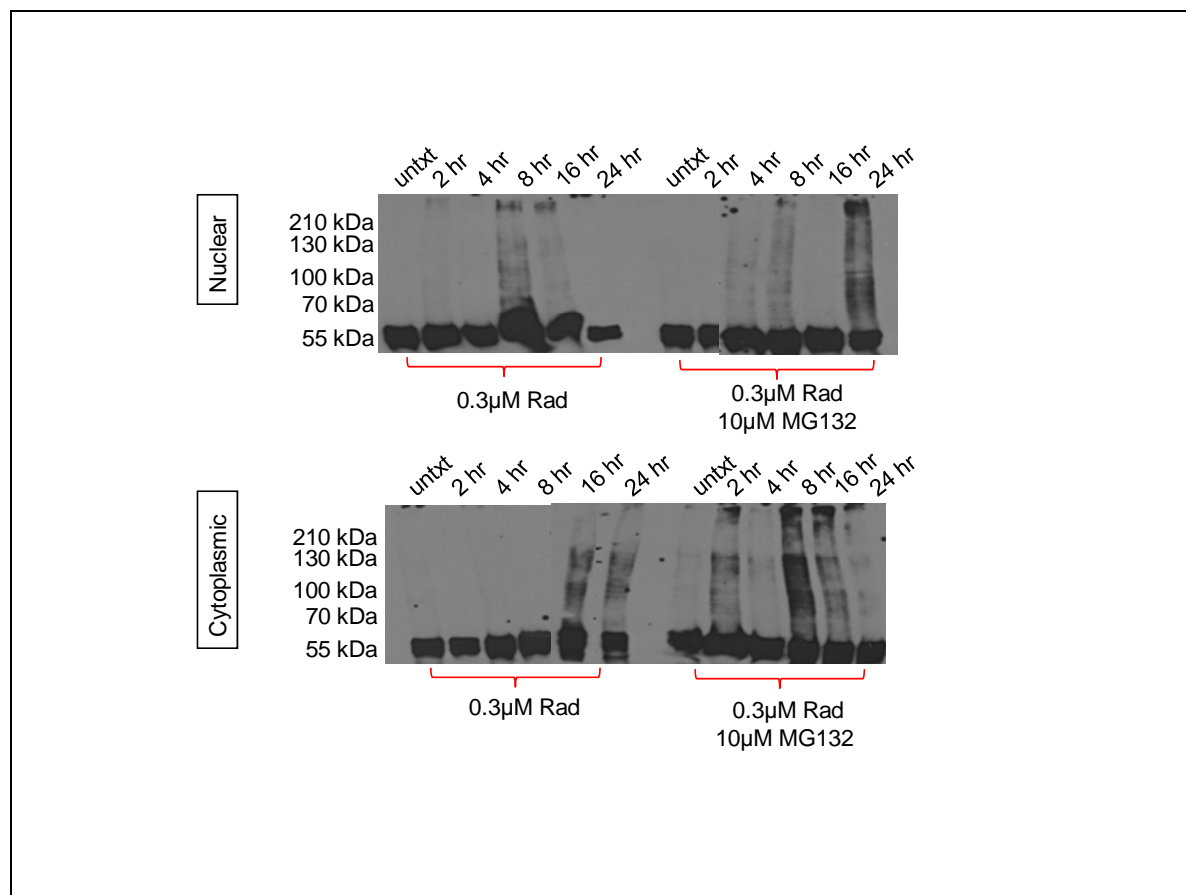


Figure 32. Inhibition of Hsp90 does not target TRF2 for ubiquitination. Cells were treated with 0.3μM RAD with or without 10μM MG132. Nuclear and cytoplasmic fractions were collected and immunoprecipitated for TRF2 and Westerns were probed for Ubiquitin. Very low intensity bands above TRF2 (65-69 kDa) indicate levels of ubiquitination as a result of normal protein turnover and likely not proteasome-mediated degradation as a result of Hsp90 inhibition.

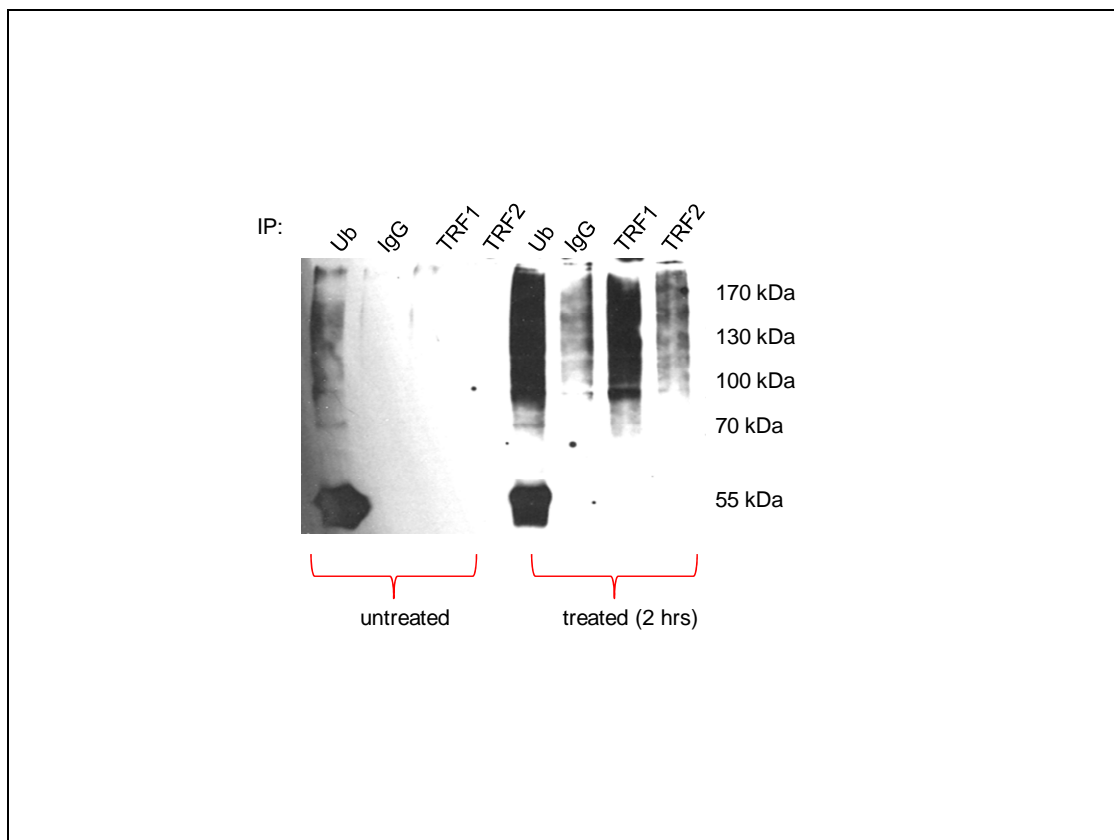


Figure 33. Inhibition of Hsp90 results in ubiquitination of TRF1. H1299 cells were treated for 2 hours with Radicicol and 10 μ M MG132 compared to untreated cells. Nuclear extracts were immunoprecipitated for TRF2, TRF1, IgG (negative control), and Ubiquitin (positive control) and Western blots were probed for Ubiquitin.

To visually confirm these results through immunocytochemistry and determine if Hsp90 targeted TRF1 for degradation, we chronically treated H1299 cells with both 0.3 μ M RAD and 10 μ M MG132. MG132 is toxic to cells at 6 hours, so we assayed for changes in colocalization out to 4 hours. When analyzed by the Leica Lite masked cytofluorogram program, colocalization between Hsp90 and TRF1 significantly increased in the nucleus at 4 hours compared to untreated ($p < 0.05$) with no evidence of TRF1 being exported to the cytoplasm for degradation (Figure 34). Therefore, the interaction between Hsp90 and TRF1 after Hsp90 inhibition does not seem to result in cytoplasmic proteasome-mediated degradation, but may be being degraded in the nucleus of tumor cells after Hsp90 inhibition.

Together, these data suggest that the Hsp90/TRF2 interaction is affected only transiently by Hsp90 inhibition with RAD. The inhibition of Hsp90 had no significant effect on TRF2 localization in the cell nor did it target TRF2 for degradation but effects telomere binding and protein-protein interactions transiently. Hsp90 may, however, play a role in TRF2's ability to associate with the telomere long-term, which could have implications for chronic Hsp90 inhibition as a means to block TRF2's telomere binding function. TRF1/Hsp90 interactions are also transiently affected by Hsp90 inhibition, and this Hsp90/TRF1 interaction may have implications in targeting TRF1 for proteasome-mediated degradation in the nucleus rather than the cytoplasm.

Radical does not induce DNA Damage

It is possible that inhibition of Hsp90 is inducing a DNA damage response at the telomere, as TRF2 is known to localize to sites of DNA damage, both telomeric and throughout the genome (Tanaka, *et al.*, 2005; Bradshaw, *et al.*, 2005; Karlseder, *et al.*, 2004; Demuth, *et al.*, 2008). We hypothesized that TRF2 was binding to Hsp90 to not only affect telomere binding or

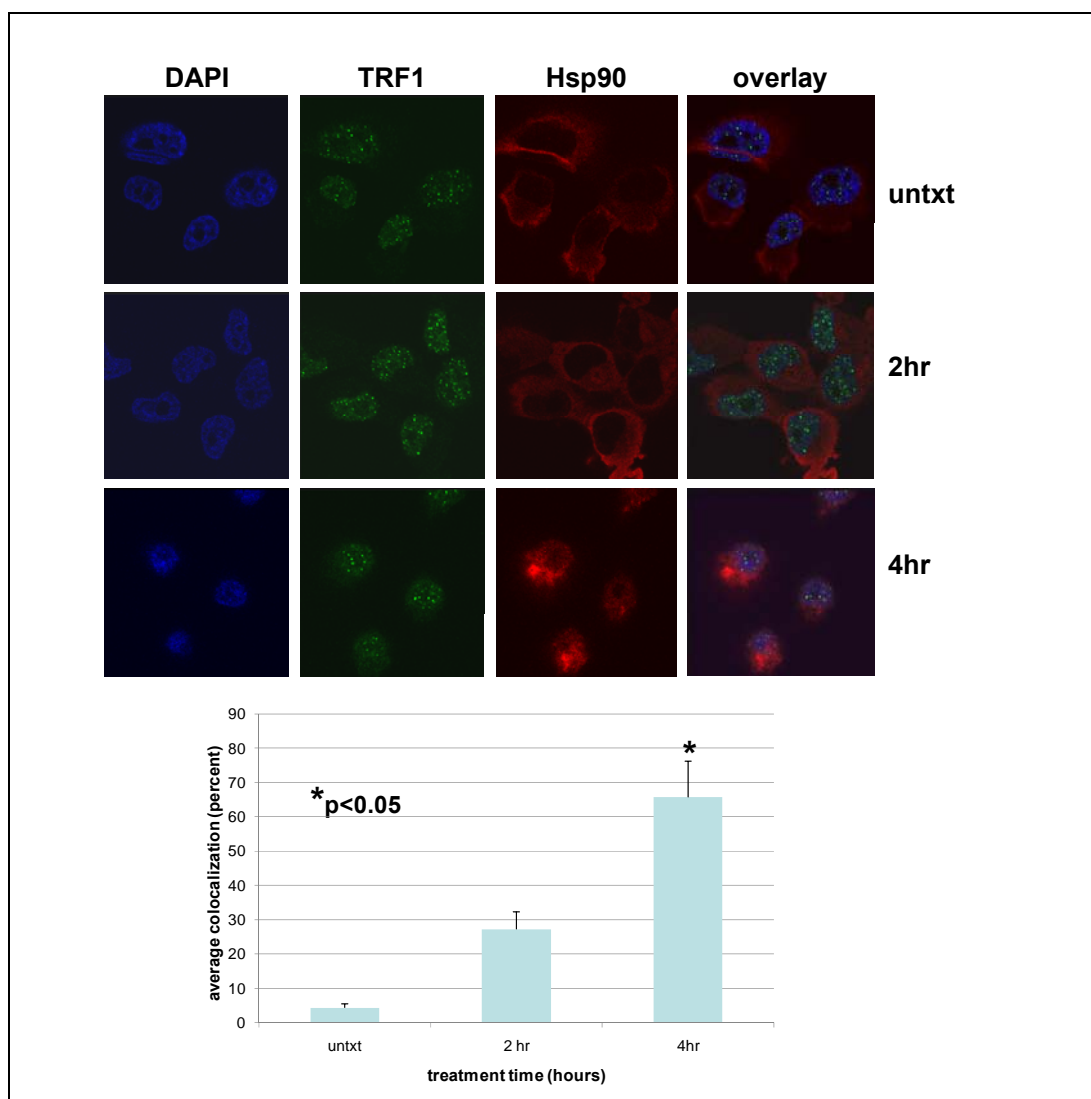


Figure 34. Inhibition of Hsp90 and proteasome-mediated degradation affects colocalization of chaperones and TRF1 in cells. H1299 cells were treated with 0.3 μ M Radicicol and 10 μ M MG132. Immunocytochemistry and analysis by Leica Lite masked cytofluorogram colocalization software of 3 fields showed significant increase in colocalization of Hsp90 (Alexa 568) and TRF1 (Alexa 488) in the nucleus at 4 hours compared to untreated (*) (Duncan's new multiple range test, $p < 0.05$). TRF1 stains the nucleus throughout treatment, indicating a lack of localization to the cytoplasm. DAPI serves as a positive control for nuclear staining.

protein-protein interactions but also in response to DNA damage or critically short telomeres. To explore this, we wanted to determine if there was an increase in telomere end-end fusions as an indicator of DNA damage. After up to 16 hours of chronic treatment with 0.3 μ M RAD, no end-end fusions were detectable using the Telomere Restriction Fragment length assay (TRF) under denaturing conditions (Figure 35B), an assay that has been used previously to detect end-end fusions (Karlseder, *et al.*, 2002). When end-end fusions occur, high molecular weight bands are observed above the normal telomeric smear (Figure 35A, 8 hour and 16 hour). No distinct bands were observed in the RAD treated H1299 samples, suggesting Hsp90 inhibition did not cause telomere dysfunction at levels detectable by the crude TRF assay.

Cytogenetic chromosomal aberrations are also an indicator of DNA damage and genomic instability. To determine if Hsp90 inhibition induced genomic instability or affected telomere structure over a longer treatment period, we examined the telomeres of metaphase chromosomes. The chromosome assessments included evaluations of the telomeric signals (location and number) and the presence of structural aberrations, with particular attention given to changes involving telomeric regions (such as end-to-end associations or terminal deletions). Although a trend toward a higher number of telomeres lacking signal was observed for the cells after exposure to RAD (when compared to controls), there appears to be no significant differences in telomere-related chromosomal changes after Hsp90 inhibition ($p > 0.05$) (Figure 36A and 36D and Table 2). Interestingly, the cells from the 3-day exposure did have a significantly increased frequency of chromosomes presenting with a “double” telomeric signal ($p < 0.05$). This unusual finding (Figure 36C) was characterized by the presence of “doublet” signals in multiple (at least two) areas of hybridization on the telomeres of the affected chromatids. However, by day 5 of treatment, the “doublet” telomeric regions were no longer observed, with no other significant

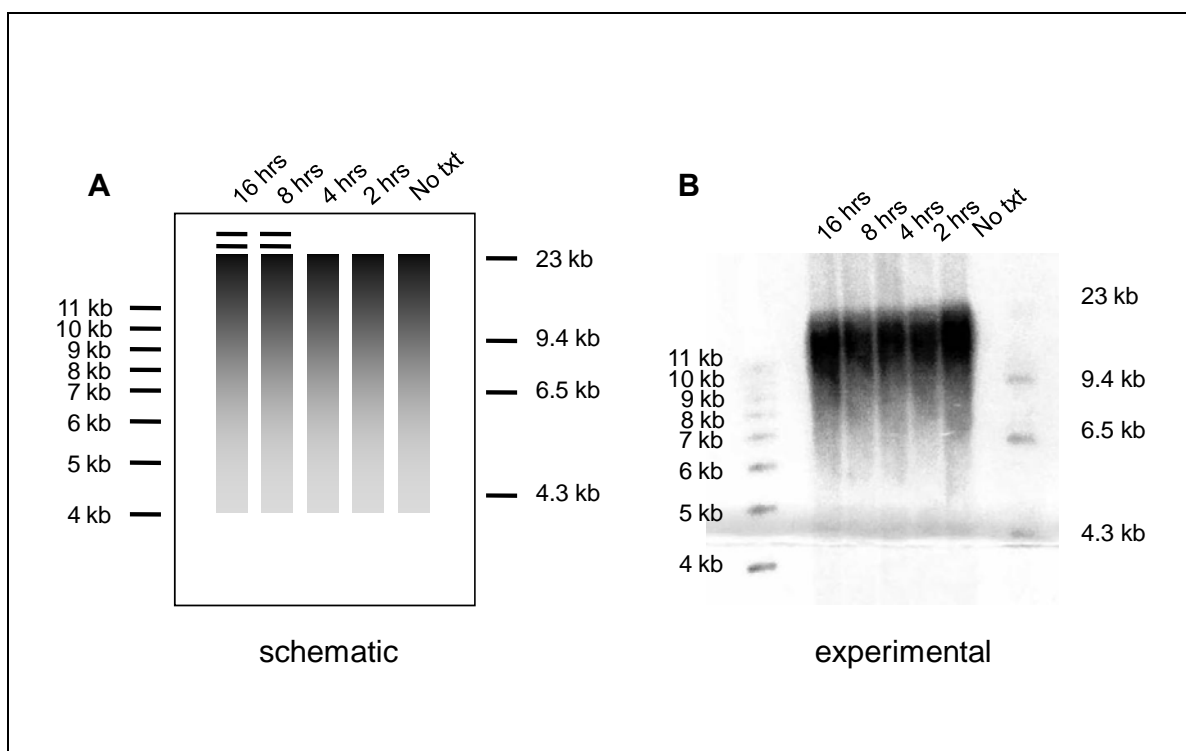


Figure 35. Hsp90 inhibition does not induce end-end fusions. **A.** A schematic representation of a Telomere Restriction Fragment length assay (TRF) in which drug treatment induces a DNA damage response, resulting in end-end fusions of chromosomes. When genomic DNA is enzymatically digested and loaded, end-end fusions cause a high molecular weight band above the telomeric smear. **B.** A TRF assay was performed after 0.3 μ M Radicol treatment for times indicated to identify telomeric end-end fusions, an indicator of DNA damage, in H1299 cells. 10 μ g of genomic DNA was enzymatically digested and loaded.

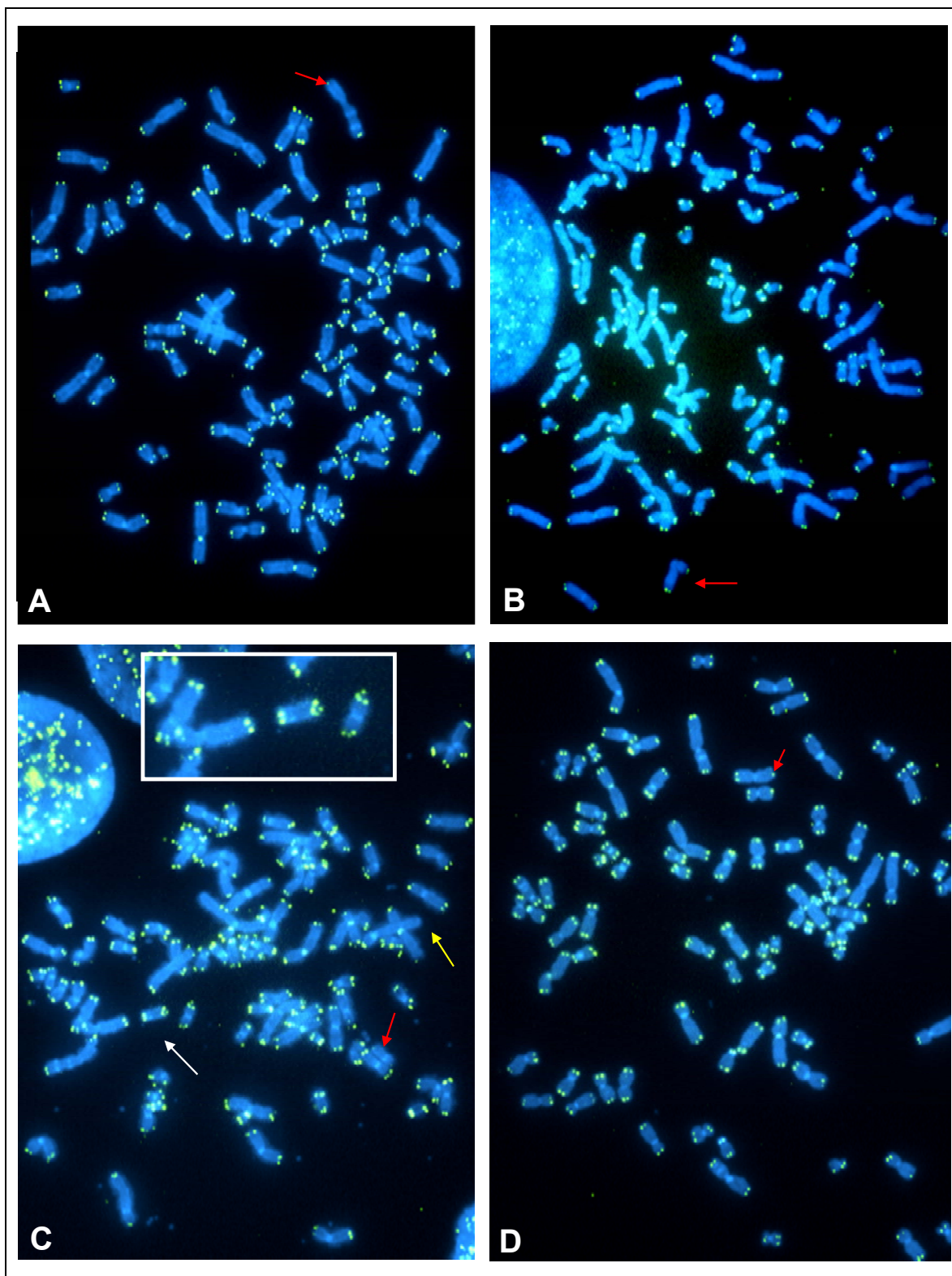


Figure 36. Representative metaphase spreads from H1299 cultures following treatment with Radicol. **A.** The baseline (untreated) metaphase spreads from this chromosomally abnormal cell line had a near-tetraploid complement. Most of the telomeres had two signals (one for each chromatid). However, 6% of the chromosomes scored had a single telomeric signal (as illustrated by the chromosome highlighted by the red arrow). **B.** The telomeric signals and chromosomal findings that were observed following 1 day of treatment with Radicol were not significantly different from those seen in the baseline culture. **C.** Following 3 days of treatment, chromosomes were observed that had one (red arrow) or no (yellow arrow) telomeric signals. Interestingly, several of the chromosomes had an apparent doubling of their telomeric signals (white arrow indicates area that is enlarged in the inset picture. The inset shows 3 chromosomes having a multi-signal telomeric appearance). **D.** After 5 days of treatment, this atypical, multi-signal telomeric appearance was no longer observed, with no other significant change in the telomeres being detected when compared to chromosomes from the baseline cultures.

Table 2. Cytogenetic findings after Hsp90 Inhibition

Metaphase spreads from treatment	Mean Xsome arms without telomere signal	Mean Xsomes with 1 chromatid telomere	Mean Xsomes with very small signal	Mean aberrations	Mean "double" signals at telomeres
Control (n=5)	1.6 (0-3)*	6.0 (2-16)	6.0 (4-9)	0.4 (0-2)	0.4 (0-1)
Day 1 (n=7)	1.3 (0-3)	5.1 (0-9)	7.0 (1-15)	0.4 (0-1)	1.3 (0-4)
Day 3 (n=7)	5.7 (1-17)	7.9 (0-18)	5.7 (1-12)	2.1 (1-6)	5.6 (2-9)[¶]
Day 5 (n=7)	1.6 (0-4)	3.9 (0-8)	4.0 (1-7)	0.1 (0-1)	0.6 (0-2)

*Mean (range/cell)

[¶] Significantly increased in cells from day 3 treatment

While there is a trend toward a higher number of chromosomes lacking a telomeric signal in the day 3 treatment this value is not significantly different [Chi-square, 3df].

differences in telomeric attributes being detected when compared to the chromosomes from control cultures. This doublet finding has been described in human cells to be associated with an induction of TIFs, growth arrest and cellular senescence (Philippe, *et al.*, 1999; van Overbeek and de Lange, 2006; Mitchell, *et al.* 2009), particularly when TRF2 is disrupted. The doublet findings here suggest that Hsp90 inhibition is, in fact, having some sort of deleterious effect on TRF2, resulting in an inability to properly protect telomeres.

Proposed model of TRF1 and TRF2 interaction with Hsp90

In this study, we show both *in vitro* and *in vivo* that TRF2 and TRF1 interact with Hsp90 and Hsp70. Additionally, Hsp90 and Hsp70 were found to interact with the telomere associated proteins, TPP1 and TIN2. The telomere is normally coated by TRF1 while TRF2 facilitates the formation of the t-loop by promoting invasion of the 3' overhang into the duplex DNA. Our results suggest that Hsp90 normally directly interacts with both TRF1 and TRF2 through a cycle of binding and unbinding to the telomere at the t-loop (Figure 37, right). Clearly, we need to investigate the role of Hsp90 in t-loop formation, but these experiments are not only costly but technically challenging, to the point where only a single lab (Griffith, *et al.*, 1999) can adequately perform these assays. That said, we have established a collaboration and are hopeful that these results will be obtained soon.

When Hsp90 is pharmacologically inhibited by Radicicol, Hsp90 comes off of the telomere inducing initial tight binding at the telomere of TRF1 and TRF2. The reason for this initial increase in telomere binding by TRF1/TRF2 after blocking Hsp90 remains unclear, but the function of most, if not all, Hsp90 client proteins does not require constant “contact” with the Hsp90 protein. The vast majority of Hsp90 target proteins only associate with chaperones in a

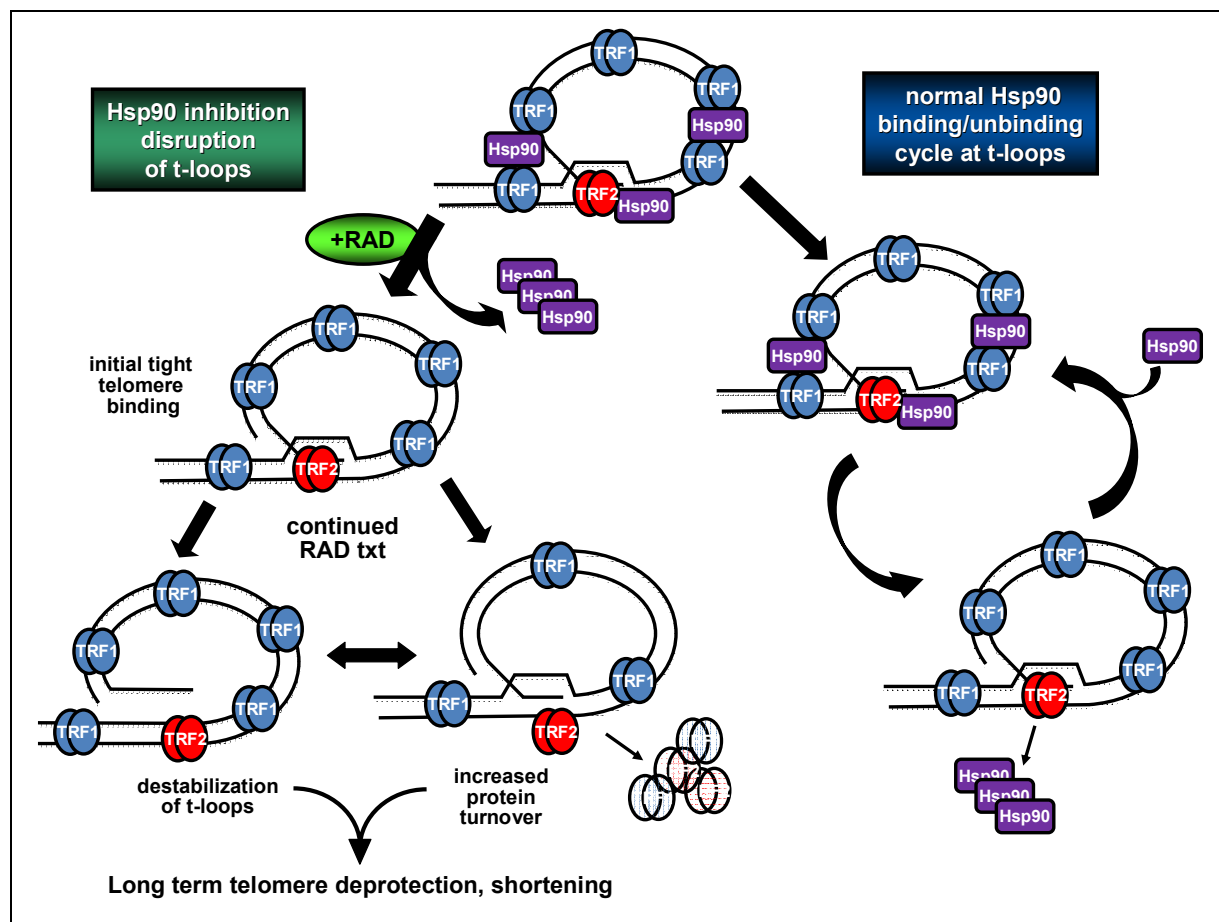


Figure 37. Model of proposed interactions of chaperone proteins at the telomere. TRF2 and TRF1 interacts with Hsp90. We propose that these two telomeric proteins normally bind to Hsp90, and Hsp90 goes through a cycle of binding and unbinding at the t-loop (right). If Hsp90 is inhibited, there is an initial tight binding to the telomere by TRF1 and TRF2. With chronic RAD treatment, there is increased protein turnover and destabilization of the t-loop, leading to long term telomere deprotection and eventual telomere shortening.

transient fashion, so the initial Hsp90 inhibition followed by an increase in TRF1/TRF2 telomere binding is consistent with previously accumulated data (Prodromou, *et al.*, 2000; reviewed in Hahn, 2009). With chronic long-term RAD treatment, there appears to be an increase in TRF1/2 protein turnover, which may be the result of TRF1 degradation (seen in Figure 33), ultimately leading to destabilization of the t-loop and long-term telomere deprotection and shortening (Figure 37, left).

Chapter 6

Discussion

The Chaperone/Telomere Interaction

When normal somatic cells divide, telomeres gradually erode until they reach a critical length. As the telomere shortens, there is less substrate for telomere binding proteins, including TRF1 and TRF2, to maintain the t-loop structure and mask the telomere from the DNA damage response machinery. This destabilization can result in the cytogenetic abnormalities of telomere dysfunction: end-end associations/fusions, anaphase bridges, radials, dicentric chromosomes, and overall genomic instability, which is a major factor in tumorigenesis. TRF2 protects the telomeres from end-end fusions and the resulting genomic instability and is required for proper telomere structure (van Steensel, *et al.*, 1998), while TRF1 coats the telomeric DNA and has been shown to prevent telomerase access to the telomere (Smorgorzewska, *et al.*, 2000). Therefore, defining the mechanisms of TRF1 and TRF2 regulation is important for both understanding the telomere and in the development of new therapeutic approaches for targeting telomeres to induce dysfunction in human tumors. One way to accomplish this may be through inhibition of the chaperone proteins, particularly Hsp90, which has been shown to be important in the treatment of many types of cancer. Clinical trials are underway employing drugs targeting Hsp90 in cancer cells (Banjeri, *et al.*, 2005; Goetz, *et al.*, 2005; Grem, *et al.*, 2005), and given the results here, these Hsp90 compounds have an effect on telomeric proteins.

Our previous studies have demonstrated that Hsp90 and p23 stably associate with telomerase, so it has been inferred that telomerase-bound chaperones will be associated with the telomere (Holt, *et al.*, 1999). In the present study, we have not only shown a specific interaction

of chaperones with telomeres, we have also identified a novel interaction between the chaperone proteins Hsp90 and Hsp70 and the telomere proteins TRF2, TRF1, TIN2 and TPP1. We speculate that this interaction may have an integral role in protection of the telomere and/or telomeric structure and the ability of telomere proteins to associate with the telomere and each other in cancer cells.

TRF2 and Hsp90

In defining the function of the chaperone/telomere association, we focused on the TRF2/Hsp90 interaction. Because there are known inhibitors of Hsp90 that are presently in Phase II clinical trials, pursuing the effect of these drugs, particularly Radicicol, provides clinical relevance to our results. As TRF2 is a major player in the protection of the telomere, this interaction may play a specific role in telomere stability and may be an attractive therapeutic target.

Pharmacologic inhibition of Hsp90 over 16 hours had no effect on TRF2 protein levels nor did it effect TRF2 localization in the cell. Hsp90 protein levels decreased with inhibition, and Hsp90 localization changed from almost exclusively cytoplasmic to both nuclear and cytoplasmic. Hsp90 levels began to return to untreated levels at 16 hours and remained stable with long term chronic treatment, up to 5 days, possibly due to an increase in association with nuclear proteins responding to the stress of chronic drug treatment. Interestingly, the interaction between Hsp90 and TRF2 was transiently disrupted, although TRF2 localization remained virtually unaffected.

As Hsp90 acts to stabilize misfolded or stressed proteins, it would be expected that it would bind to those proteins in both the cytoplasm and the nucleus and remain bound until

targeted for degradation. We initially hypothesized that Hsp90 was interacting with TRF2 as a means to target it for ubiquitination and degradation. In previous studies of the effects of Radicicol and Geldanamycin, Hsp90 client proteins are most often targeted for proteasome-mediated degradation in the cytoplasm after treatment (reviewed in Messaoudi, *et al.*, 2008). However, when treated with Radicicol, TRF2 remained stably localized in the nucleus and no increase in association with Ubiquitin was observed. To ensure that TRF2 was not being degraded prior to sample collection, treatment with the proteasome inhibitor MG132 verified that TRF2 remained in the nucleus after treatment and was not being exported to the cytoplasm as a means for degradation, suggesting the functional association of Hsp90 and TRF2 is therefore not based upon Hsp90 targeting TRF2 for degradation. Any ubiquitination of TRF2 observed appears likely the result of normal protein turnover, not due to the Hsp90/TRF2 interaction being inhibited through Radicicol treatment that occurs with other Hsp90 client proteins such as ErbB2 and mutant p53 (reviewed in Messaoudi, *et al.*, 2008).

Although pharmacologic inhibition of Hsp90 did not affect TRF2 protein levels or localization, it did seem to effect the interaction between Hsp90 and TRF2 and the association of TRF2 at the telomere. When treated chronically over 16 hours, the interaction between TRF2 and Hsp90 decreased significantly at 8 hours and returned to low levels at 16 hours. The interaction remained highly nuclear throughout treatment with only background association detectable in the cytoplasm. When we assessed TRF2's ability to bind to the telomere (through ChIP and colocalization with TRF1, a second telomere binding protein as a marker of telomere colocalization), we observed a significant increase in TRF2's association with telomeric sequence at 2 hours and 4 hours of Radicicol treatment when compared to controls, even though the Hsp90/TRF2 interaction was blocked. At 8 hours, TRF2 binding was diminished to levels

similar to untreated controls. Immunocytochemistry (ICC) showed a significant decrease in TRF2/TRF1 colocalization at 8 hours of RAD treatment, which may reflect an effect of Hsp90 inhibition on TRF1 rather than TRF1/TRF2 colocalization. ImmunoFISH experiments would be ideal to verify if Hsp90 inhibition is indeed affecting TRF2's ability to bind the telomere. It appears that inhibition of Hsp90 initially caused elevated binding of TRF2 to the telomere, which decreased with continued inhibition of Hsp90.

These results suggest two possible roles for the Hsp90/TRF2 interaction: (1) Hsp90 interacts with TRF2 to facilitate TRF2's binding to the telomere, acting as a conformational bridge for proper telomere binding, possibly associating with TRF2 near its DNA binding domain. When inhibited, Hsp90 is removed from the complex, leaving a properly folded TRF2 that is capable of strongly binding to telomere, resulting in an increased TRF2 telomere signal. However, at later time points, TRF2 appeared to be unable to stably associate with the telomere, suggesting that TRF2 needs Hsp90 to bind to telomeres long-term. A determination of where Hsp90 binds to TRF2 may still provide clues as to the functional role between Hsp90 and TRF2. If Hsp90 binds at or near the DNA binding domain of TRF2, it is possible that Hsp90 is assisting but is not absolutely required for TRF2's binding to telomeric sequence, which may have implications in TRF2's ability to bind elsewhere and its function in the DNA damage response.

The Hsp90/TRF2 interaction may play another possible role: (2) Hsp90 may stabilize TRF2 at the telomere, acting solely on TRF2 to maintain telomere structure rather than bridging the telomere and TRF2. In this scenario, Hsp90 may have an effect on protein turnover, and when Hsp90 is inhibited, TRF2's half-life at the telomere decreases in the long term. Although we did not observe an increase in TRF2 ubiquitination, it may be that the rate of turnover is increased independent of any sort of post-translational modification.

TRF2 is intricately involved in the DNA damage response, both telomerically and globally (Tanaka, *et al.*, 2005; Bradshaw, *et al.*, 2005; Karlseder, *et al.*, 2004; Demuth, *et al.*, 2008). It is the only Shelterin protein to bind to numerous DNA damage proteins found at the telomere, including the Mre11 complex, and so far, is the only Shelterin protein implicated in sensing DNA damage elsewhere (Karlseder, *et al.*, 1999; Bradshaw, *et al.*, 2005). However, Radicicol treatment did not induce a DNA damage response, consistent with what has been observed previously (Compton, *et al.*, 2006). We looked for an induction of end-end fusions with treatment, which are indicative of a DNA damage response (van Steensel, *et al.*, 1998), but a denaturing TRF assay revealed no induction of telomere fusions over the 16 hour treatment time.

Cytogenetic examination of metaphase spreads over 5 days of chronic treatment with Radicicol also revealed no significant increase in loss of telomere signals or structural aberrations that would suggest increased DNA damage relevant to TRF2 and/or Hsp90. However, at day 3 of treatment there was an increase in a “doublet” telomeric signal. The presence of telomeric doublets suggests a Radicicol-induced effect on TRF2, similar to that seen previously (Philippe, *et al.*, 1999; van Overbeek, *et al.*, 2006; Mitchell, *et al.* 2009), where an absence of structural chromosomal aberrations but an induction of TIFs was also observed. One could speculate that this atypical, doubling of the telomeric area resulted from a change in the telomeric chromatin (possible break between regions or duplication of the region) as a result of disrupting either the TRF2/Hsp90 or TRF1/Hsp90 interaction. The Hsp90/TRF2 interaction may occur so that TRF2 is able to bind to the telomere “loosely” to allow for t-loop formation and telomere chromatin unwinding. When the normal Hsp90 interaction is inhibited, the lack of association potentially triggers an incomplete DNA damage response involving only TRF2 and promotes tight telomere binding of TRF2 to aid in its function of telomere capping and

prevention of loss of the t-loop early (2-4 hours). Long term inhibition of Hsp90 (3 days) results in a change in telomeric chromatin that alters TRF1/TRF2/telomere binding, thus altering the chromatin structure itself. Examination of the t-loop and TRF2's ability to bind and induce strand invasion before and after Hsp90 inhibition may provide conclusions to this hypothesis (Stansel, *et al.*, 2001). Even though these results are interesting considering TRF2 has been implicated in chromatin remodeling (Amiard, *et al.*, 2007), further examination of the TRF2/Hsp90 interaction and its effects on telomeric chromatin and t-loop structure is underway with our collaboration with Dr. Sarah Compton at UNC Chapel Hill.

Although the effects seen in this study were conducted mainly in the H1299 lung carcinoma cells, the interaction between Hsp90 and TRF2 also was found in MCF7 breast cancer cells, M12 prostate cancer cells, and BJ fibroblast cells (a normal cell culture). Therefore, it is likely that the effects observed here occur universally in normal and cancer cell lines. It appears that the Hsp90/TRF2 complex is a normally occurring interaction, not influenced by dysfunctional or critically short telomeres found in cancer cells. If Hsp90 does, in fact, interact with TRF2 as a means to facilitate TRF2 telomere binding, understanding this relationship is imperative to understanding how Hsp90 and TRF2 function in the cell. As Hsp90 inhibitors like 17-AAG have entered into clinic trials for use as adjuvant treatment for breast cancer and prostate cancer, it is important to determine how this inhibition effects TRF2's role in telomere structure and function, as disruption of this interaction could effect a vital telomere protective factor. A better understanding of both TRF2 and Hsp90 and their roles in nuclear events is necessary, as both have extremely important functions in the cell.

TRF2 and Hsp70

Hsp70 and TRF2 were also found to interact in H1299, MCF7, and BJ cells. RAD treatment resulted in a decrease in Hsp70 protein levels earlier than Hsp90, with a return to higher levels than Hsp90 at 16 hours. A significant decrease in Hsp70 binding to telomere sequence occurred after Hsp90 inhibition when compared to Hsp90. This was all expected after Hsp90 inhibition, as Hsp70 complexes with Hsp90 (Wegele, *et al.*, 2004). RAD treatment would be expected to result in a similar, if not more dramatic inhibition of Hsp70 because it is likely indirectly associating with the telomere, and hence telomere binding proteins, through Hsp90.

When treated with Radicicol (with or without MG132), there was no detectable cytoplasmic interaction between Hsp90 and TRF2; however the cytoplasmic interaction between Hsp70 and TRF2 was significantly increased, particularly at 8 hours. From this, it could be hypothesized that TRF2 is binding to Hsp70 to be exported to the cytoplasm for ubiquitination. However, there was no increase in TRF2 ubiquitination or cytoplasmic localization after Radicicol treatment, suggesting that the interaction observed between Hsp70 and TRF2, especially in cytoplasmic extracts, was merely an artifact of Co-IP analysis. It is likely that Hsp70 is immunoprecipitating with TRF2 through its association with Hsp90, not through a direct association between Hsp70 and TRF2.

TIN2, TPP1 and Hsp90

Early inhibition of Hsp90 resulted in a significant increase in TIN2 and TPP1 binding to telomeric sequence, similar to that found for TRF2 and TRF1. TIN2 interacts with both TRF1 and TRF2, forming a bridge between the two proteins, while TPP1 binds to TIN2. Although neither TIN2 nor TPP1 interact directly with telomeric DNA, as TRF1 and TRF2 do, the

increased association of protein and telomeric sequence suggests that Hsp90 may play a role in TIN2 and TPP1 regulation similar to its role with TRF2: facilitating association between the telomere and binding proteins. Radicicol treatment had a transient effect on TRF2/TIN2 interaction implying that Hsp90 may contribute to the protein-protein interactions of the Shelterin complex. The TRF2/TPP1 interaction appeared to be enhanced significantly after Hsp90 inhibition, which is interesting because these proteins do not directly interact, suggesting a role for Hsp90 in mediating telomere protein-protein interactions.

TIN2 and TPP1's novel association with Hsp90 and Hsp70 shows that Hsp90 may be interacting with all Shelterin proteins, including TRF2, TIN2, TPP1 and TRF1, all with similar effects seen on telomere binding after Hsp90 inhibition. These results suggest that the chaperones, Hsp90 in particular, play a role in facilitating telomere associated protein/telomere binding and/or telomere protein-protein interactions.

TRF1 and Hsp90/Hsp70

As evidenced by the increase in TRF1's telomere binding after Radicicol treatment and the interactions of TRF2, TIN2 and TPP1 with the chaperones, it was logical to examine TRF1's interaction with Hsp90 and Hsp70 and how inhibition of Hsp90 affects TRF1. The interaction between Hsp90/TRF1 and Hsp70/TRF1 was effected by Radicicol treatment, although it differed from the results seen with TRF2. TRF2/chaperone interactions were greatly diminished at 8 hours, while the TRF1/Hsp90 and TRF1/Hsp70 interactions were almost undetectable at 2 hours, returning consistently at the highest levels at 8 hours. As opposed to TRF2 where we saw no induction of ubiquitination, TRF1 appears to be substantially ubiquitinated after just 2 hours of RAD treatment. There was, however, no exportation to the cytoplasm, suggesting against a

cytosolic proteasome-mediated degradation pathway and for a nuclear degradation mechanism (von Mikecz, 2006). The disruption of TRF1/TRF2 colocalization with RAD treatment further suggests that inhibition of Hsp90 disrupts TRF1 function in the nucleus.

The significant increase in chaperone/TRF1 interaction at 8 hours, which was so reduced at 8 hours for TRF2, may be the result of the chaperones “hopping” from one telomeric protein to the other due to an increased need for stabilization after treatment. Although both TRF1 and TRF2 act as protective factors at the telomere, they do so in different ways and, if repressed, present with differing consequences. Both TRF1 and TRF2 act as negative regulators of telomere length, repressing telomerase activity in cells. When TRF1 is repressed, telomere length increases as telomerase is able to extend telomeric repeats. When TRF2 is repressed, the G-strand overhang shortens and there is an upregulation of DNA damage factors associating with the telomere (reviewed in: De Boeck, *et al.*, 2009). The increase in telomere binding, along with the interactions seen between Hsp90 and TRF2 at 2-4 hours, suggests an early need for TRF2 to bind and protect the telomere (possibly as a means to protect the t-loop), as well as for responding to DNA damage events. The later upregulation of the Hsp90/TRF1 interaction may be the result of an increased need to prevent telomerase from binding to the telomere or to stabilize the telomere structure in order to prevent telomere degradation by nucleases.

The observed difference between the TRF1/Hsp90 and TRF2/Hsp90 interactions could be affected by the differing half-life times of TRF1 and TRF2. It has been suggested that mammalian telomeres employ a protein counting mechanism as increased accumulation of TRF1 and TRF2 at the telomere results in telomere shortening (Smorgorzewska, *et al.*, 2000; van Steensel, *et al.*, 1997). This is based on observations in yeast that there are a set number of TRF1/TRF2 binding sites and insertion of artificial binding sites results in telomere shortening

(Marcand, et al., 1997; Ancelin, et al., 2002). Photobleaching experiments showed that telomere length is maintained by dynamic binding of TRF1 and TRF2 with binding times of only 8 seconds, so that the telomere is able to adapt quickly to telomerase or nuclease actions (Mattern, et al., 2004). TRF2 also has a “slow fraction” (27%) that binds for 11 minutes, which has been proposed to facilitate t-loop formation and stabilization. In fact, it has been shown that this slower fraction binds more stably to the telomere. When coupled with the “fast fraction”, it is suggested that TRF2 associates with telomeres in two complexes: one with a role in telomere length regulation and one regulating chromosome end protection (Mattern, et al., 2004). Hsp90 interactions may be mediating the dynamic telomere binding of TRF2. It is possible that the TRF2 slow fraction is interacting early with Hsp90 and at 8 hours is lost; whereas, all TRF1 is continuously and quickly turning over, and the TRF1/Hsp90 interaction is effected much earlier. The longer the Hsp90 inhibition, the more disrupted the ability of the telomere binding protein (TRF1 or TRF2) to bind to the telomere, resulting in the decrease in telomere binding with chronic treatment.

There may be a cell cycle-dependence for these interactions; however, because all Shelterin proteins are bound to or associated with the telomere throughout much of the cell cycle, this seems unlikely. There is also the possibility that the increased chaperone/TRF1 and TRF2 interactions are effects of telomere protein-protein interactions. For example, TRF2 may bind stably to Rap1 in an immediate response to drug treatment, but TRF1/POT1’s interactions may need to be stabilized over longer periods of time, with Hsp90 facilitating these associations. The TIN2/TPP1/Hsp90 association may be evidence of this, as TPP1 tethers POT1 to TRF1 and TIN2 tethers TPP1 to TRF1 and TRF2 (reviewed in: De Boeck, et al., 2009), all of which may be mediated by Hsp90.

TRF2 has been identified as having different roles in chromatin remodeling where overexpression of TRF2 resulted in a decrease in histones H3 and H4 and subsequent disruption of nucleosomal spacing specific to telomeric chromatin (Benetti, *et al.*, 2008). TRF2 can generate positive supercoiling with an ability to condense DNA. TRF2 wraps the DNA around itself and induces the untwisting of nearby DNA, allowing for strand invasion by positive supercoiling, as what is proposed to occur when forming the t-loop (Amiard, *et al.*, 2007). TRF1 also alters nucleosome structure by recognizing binding sites on telomeric nucleosomes and binding those sites through its TRFH DNA binding domain, which effects DNase I digestion patterns and indicates alterations in nucleosome core structure (Galati, *et al.*, 2006). Therefore, the “doublet” FISH signal observed in day 3 Radicicol treated cells may be the result of either (or both) TRF1/TRF2 effecting telomeric chromatin structure, again implying that the chaperone/telomere binding protein interaction is dependent on an association with telomeric sequence and effects the ability of binding proteins to associate and function stably at the telomere.

Summary

Several telomeric proteins regulate telomere length, and shortened telomeres have been shown to be a marker for cancer (Griffith, *et al.*, 1999; Odagiri, *et al.*, 1994). With a shorter telomere and fewer associated telomere binding proteins, telomeres may be an ideal target for cancer treatment. In this work, we present novel findings that the chaperone proteins Hsp90 and Hsp70 interact with the telomere proteins TRF2, TRF1, TIN2 and TPP1, suggesting that we may be able to target telomeres via chaperone inhibition.

Our initial hypothesis was that chaperone proteins interact with the telomere independent of telomerase; therefore, the goal of this study was to elucidate the role of chaperones in both telomere structure and function by defining the relationship between the chaperones and telomeric proteins. We first identified chaperones Hsp90, Hsp70 and p23 bound to telomeric DNA through ChIP. We hypothesized that the chaperones were interacting with telomeric proteins, which was verified through Co-IP/Westerns with TRF1, TRF2, TIN2, and TPP1. Through pharmacologic inhibition of Hsp90 using Radicicol, we sought to define the functions of these interactions initially postulating that Hsp90 bound to TRF1 and TRF2 as a means to target the proteins for degradation. With chronic hsp90 inhibition, we found a transient change over time in telomere binding capability for TRF1 and TRF2 as well as a transient change in the Hsp90/TRF1 and Hsp90/TRF2 interactions over time. However, when examined by immunocytochemistry and Western there was no change in telomere protein localization or levels. Interestingly, RAD treatment resulted in an increase in TRF2/TPP1 interaction, two proteins that do not normally directly interact. No evidence of drug-induced ubiquitination was observed for TRF2, yet TRF1 did appear to be ubiquitinated and potentially degraded in the nucleus rather than the cytoplasm. These results suggest an important role for Hsp90 in telomeric protein-protein interactions and overall telomere structure and function.

Because telomere associated proteins are so critical in maintaining genetic stability, an interaction with a stress protein such as Hsp90 may be an indicator that there are recurrent problems at chromosome ends that require a stress response. Alternatively, Hsp90 at the telomere may be required for continuously stabilizing proteins at the telomere, and continued inhibition may result in an inability of TRF1/TRF2 to properly bind the telomere resulting in telomeric deprotection and eventual genomic instability and senescence/apoptosis. Additionally,

the use of Hsp90 inhibitors in cancer treatment suggests a need to fully understand the mechanisms of action of these drugs. A study of Hsp90's telomeric functions in cancer cells provides a better understanding of the role of current Hsp90 inhibitors and their mechanisms of action on the chromosome structure. The novel interactions between chaperones and telomere associated proteins identified here opens the door for a new area of study in telomere biology, and further understanding the role of these interactions in telomere protection may lead to additional targets for cancer therapy.

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Appendix

Appendix

Post-Translational Modification of TRF2 through SUMOylation

INTRODUCTION

Post-translational modification of telomeric proteins has not been widely studied, although a number of protein kinases associate at the telomere to aid in telomere maintenance, including ATM and DNA-PKcs (d'Adda di Fagagna, *et al.*, 2004). While many telomeric proteins have been shown to be phosphorylated or ubiquitinated, little has been reported with regard to TRF2 post-translational modifications.

SUMOylation is a form of post-translational modification with a pathway similar to ubiquitin, although its roles differ significantly from ubiquitin. SUMOylation is involved in protein-protein interactions, DNA binding, protein localization, and trafficking, as well as facilitating degradation. Recent evidence suggests that SUMOylation may have a role in Alternative Lengthening of Telomeres (ALT) cells, which employs homologous recombination rather than telomerase to lengthen telomeres, with some evidence for TRF2/SUMO alteration (Potts and Yu, 2007; Zhao and Blobel, 2005; Xhemalce, *et al.*, 2007). Interestingly, there is no published evidence of Shelterin protein SUMOylation in telomerase-positive cells.

We hypothesized that TRF2 is SUMOylated in all cells. In an effort to determine if this occurs in telomerase-positive cells as well as determine the functional significance of this modification, various TRF2 fusion proteins and mutants were generated to examine the role SUMO plays in regulating TRF2. TRF2 is an important protein at the telomere whose main function is to protect the telomere ends, preventing telomere dysfunction and genomic instability.

Elucidation of the regulation of TRF2 will provide a better understanding of its role in telomere biology and protection.

RESULTS

SUMOylation of TRF2

Because SUMOylated proteins are involved in DNA binding and TRF2 is a telomeric-DNA binding protein, SUMOylation of TRF2 may have major implications in its ability to perform its functions. Potential SUMOylation sites in the TRF2 amino acid sequence were identified using the Abgent SUMOPlot program (Figure 38). Possible SUMOylation consensus sequences (ψ KxE) were predicted, and those with the highest probability were identified for further examination. The sites that are removed in the DNTRF2 sequence were also identified to ensure that no high probability SUMOylation sites chosen were contained in the eliminated N- or C- terminus because it was hypothesized that both TRF2 and DNTRF2 were SUMOylated, as the sites with the highest probability were located within the DNTRF2 sequence (Figure 39).

The two predicted SUMOylation sites identified and scored with the highest probability of being TRF2 SUMOylation sites (Figure 38) were chosen for further examination of their potential role in TRF2's post-translational regulation (Figure 41). Site-directed mutagenesis was performed to determine how un-SUMOylated TRF2 functions in the cell. The two positions chosen were at the lysine (K) at amino acid 140 (sequence: IKTE), located at 442-444 bp, and amino acid 245 (sequence: LKSE), located at 857-859 bp (Figures 38, 39 and 40). The lysine codon AAA was mutated to an arginine (R) codon AGA for both sites, as previously reported (Potts and Yu, 2007). The results of previous studies have also suggested that a double mutation

No.	Position	Group	Score
1	K140	NVLEM IKTE FTLTE	0.94
2	K245	MAKKA LKSE SAASS	0.91
3	K307	SEAAF AKLD QKDLV	0.79
4	K459	EESEW VKAG VQKYG	0.76
5	K255	AASST GKED KQPAP	0.67
6	K405	PGEKN PKVP KGKWN	0.50
7	K33	KNKRP RKDE NESSA	0.44
8	K267	APGPV EKPP REPAR	0.39
9	K258	STGKE DKQP APGPV	0.39
10	K402	QPLPG EKNP KVPKG	0.39
11	K464	VKAGV QKYG EGNWA	0.33

} Chosen for site-directed mutagenesis

Figure 38. SUMOylation sites chosen to mutate for site-directed mutagenesis. Using the Abgent SUMOPLLOT program, the possible TRF2 SUMOylation sites were given scores. The two highest scoring possible sequences were chosen for site-directed mutagenesis at position K140 and K245. The lysine at K140 (IKTE) was mutated from the amino acid sequence AAA to and arginine, sequence AGA. The lysine at K245 (LKSE) was also mutated to an arginine (AAA to AGA).

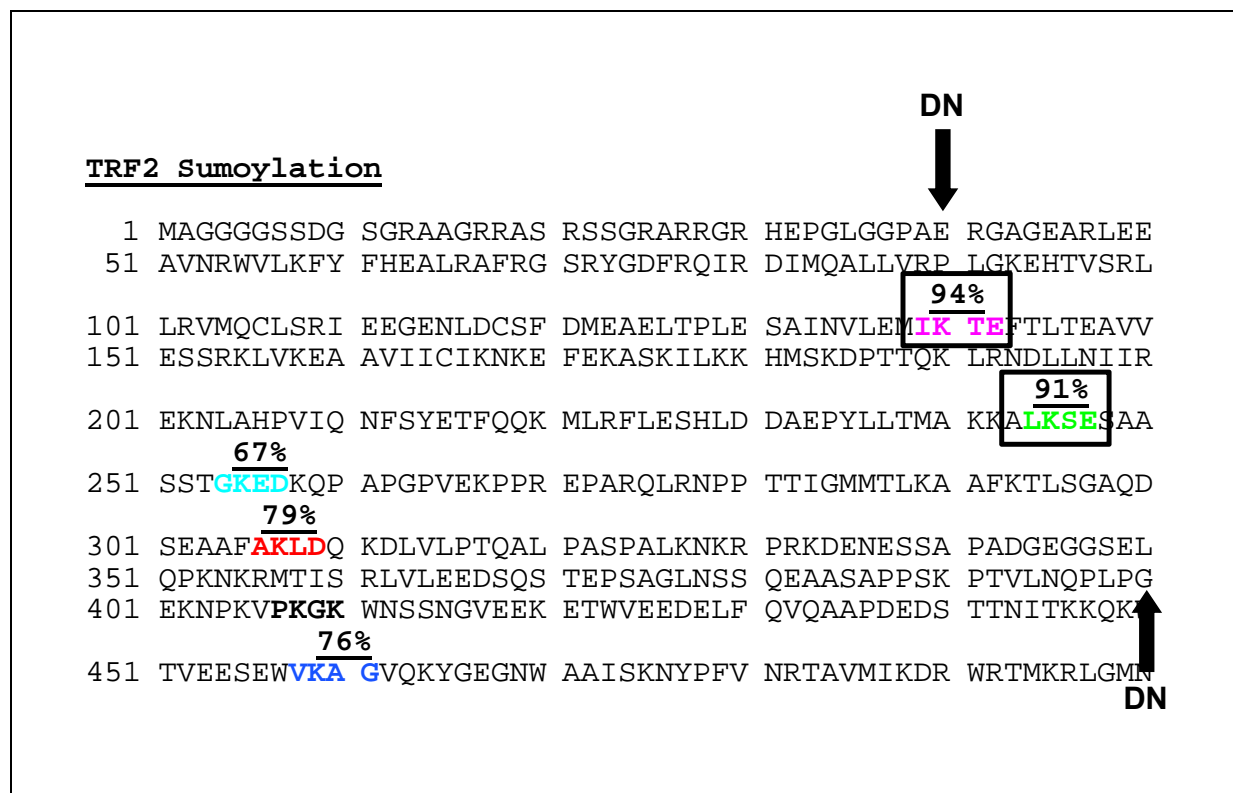


Figure 39. Predicted SUMOylation sites of TRF2. Using the Abgent SUMOPLLOT program, predicted SUMOylation sites in the TRF2 amino acid sequence were found. Two with the highest probability are shown boxed while 3 others are highlighted. The sites where the DNTRF2 amino acid sequence stops and starts are indicated by arrows.

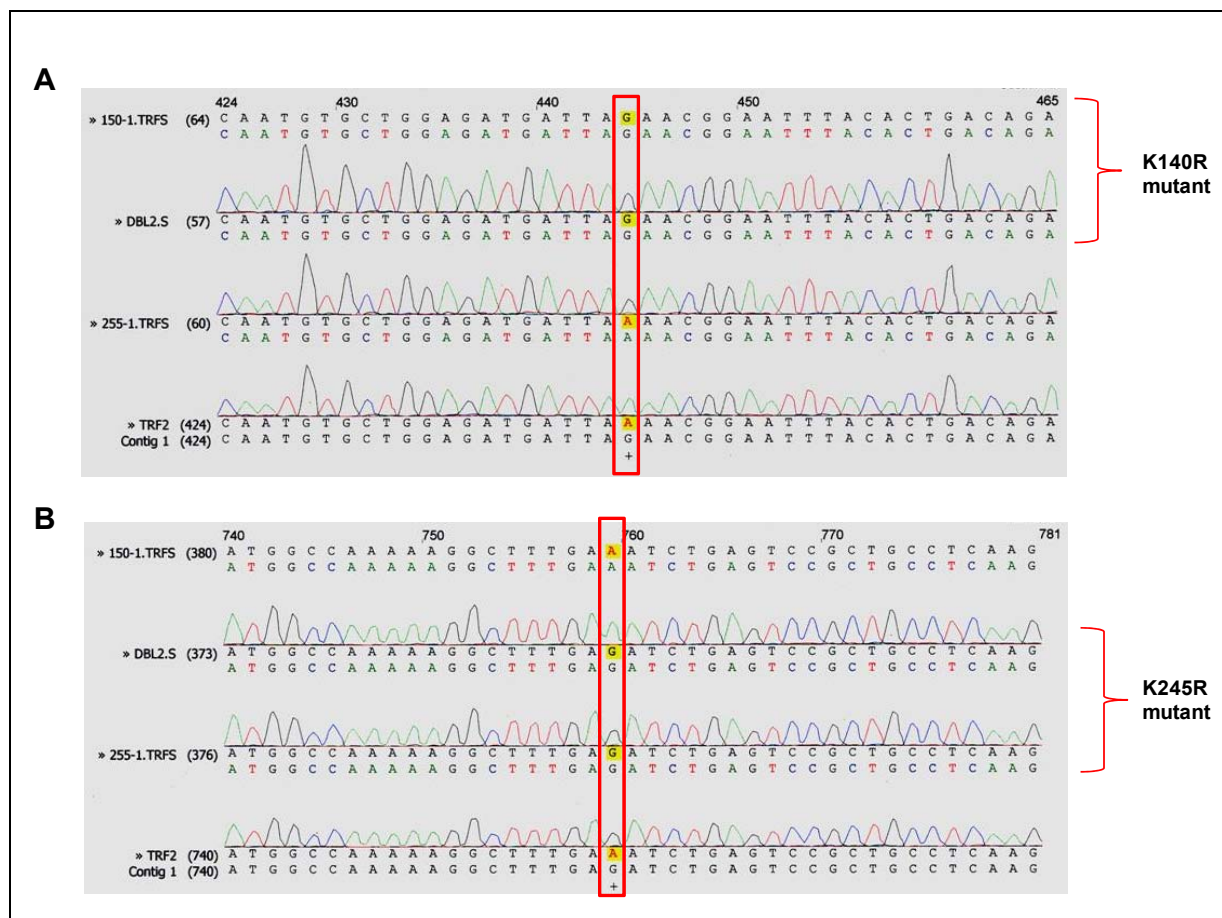


Figure 40. Sequence verification of TRF2 site-directed mutagenesis. A. Chromatogram of original sequence of K140 compared to the mutation. Highlighted base pair indicates mutation of A to G, changing the amino acid sequence from lysine to arginine. **B.** Chromatogram of original sequence of K245 compared to mutation. Highlighted base pair indicates mutation of A to G, changing the amino acid sequence from lysine to arginine.

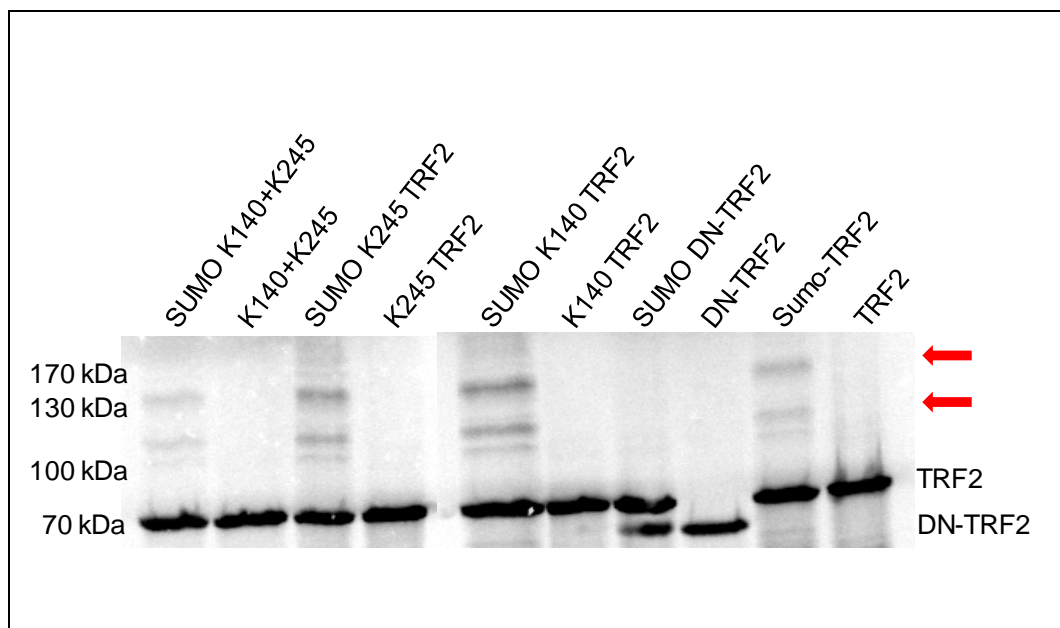


Figure 41. *In vitro* SUMOylation of TRF2. TRF2, DN-TRF2, mutant K140 TRF2, mutant K245 TRF2 and double mutant K140/K245 TRF2 were synthesized using the ^{35}S labeled RRL transcription translation (TnT) system. Samples were then assayed for SUMOylation using the Vaxron *In Vitro* SUMOylation Assay kit in the presence of SUMO-1 and SUMOylation enzymes Ubc9 and SAE1/SAE2. SUMOylated samples were run next to unSUMOylated samples with TRF2 serving as a positive control and DN-TRF2 serving as a negative control.

is required for complete loss of SUMOylation of telomeric proteins, as a single mutation of a SUMO binding site is not sufficient (Potts and Yu, 2007). Therefore, we also created a SUMO double mutant at amino acids 140 and 245 with both lysines mutated to arginines. The TRF2 plasmid, pcDNA3-TRF2full, was used in the Stratagene QuikChange II Site-Directed Mutagenesis Kit and sequenced (Figure 40).

To determine if TRF2 is SUMOylated, we first examined *in vitro* synthesized TRF2's SUMOylation. Both TRF2 and DNTRF2 were synthesized using the RRL system and then assayed for SUMOylation with the Vaxxon SUMOylation kit. Samples were synthesized in the presence of the SUMO protein SUMO1 as well as the SUMO activating enzymes SAE1/SAE2 and the SUMO conjugating enzyme Ubc9. SUMO adds 11 kDa to the protein and the bands above the TRF2 band (at approximately 69 kDa) indicate TRF2 and not DNTRF2 is SUMOylated (Figure 41). Two repeated experiments confirm that TRF2 is SUMOylated (data not shown). A possible SUMOylation site with moderate probability (76%) was located outside of the DNTRF2 sequence, and DNTRF2 appears to not be SUMOylated (Figure 38, Figure 39, Figure 41), suggesting an important site to be explored further. To test the site-directed mutations *in vitro*, samples were synthesized using the RRL system and assayed with the Vaxxon SUMOylation kit. Unexpectedly, only the double mutant resulted in a modest loss of TRF2's SUMOylated bands (Figure 41). Further experiments involving transfection of the double mutant into telomerase positive cells to examine effects on TRF2's localization, protein-protein interactions, and degradation are currently being conducted.

To confirm that TRF2 is SUMOylated in telomerase positive cells, MCF7 cells were treated with N-Ethylmaleimide (NEM), which prevents SUMO proteases from removing the SUMO moiety from the target protein after cell lysis. Whole cell extracts were

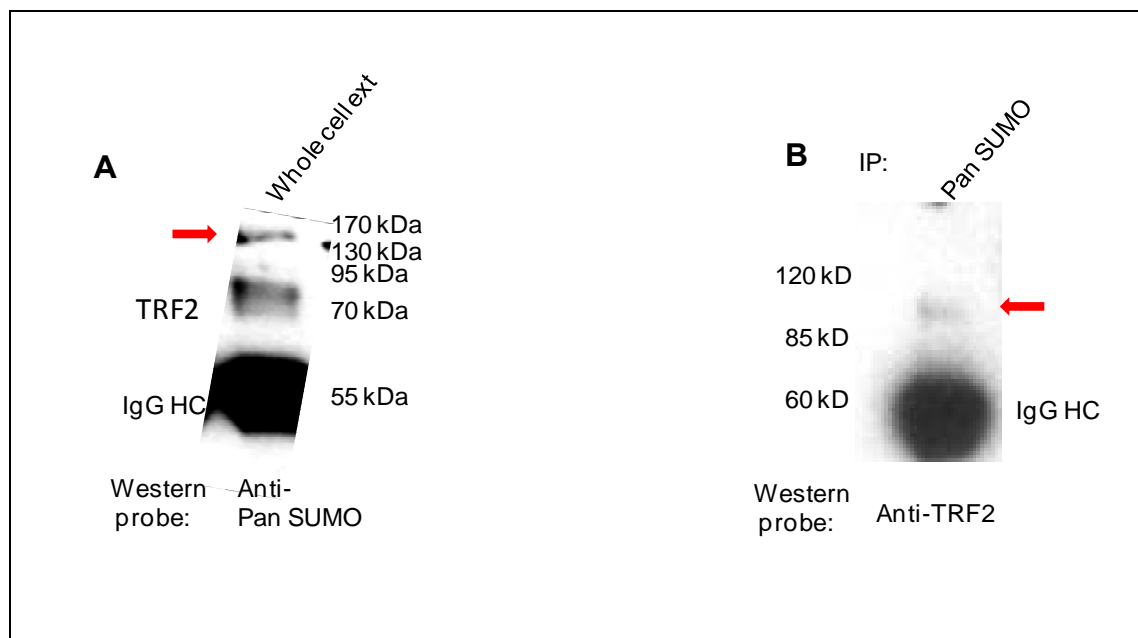


Figure 42. In cell SUMOylation of TRF2. Whole cell extracts were processed from MCF7 cells in the presence of the chemical NEM, which prevents SUMO proteases from cleaving the SUMO off proteins during the extraction process. Samples were then immunoprecipitated for TRF2 (**A**) or the Pan SUMO antibody (**B**) (which recognizes SUMO1 and SUMO2/3). Western blots were probed as indicated. TRF2 migrates at 65/69 kDa, the higher bands indicate SUMO modification of TRF2 (**A** and **B**).

immunoprecipitated for TRF2 or PAN-SUMO, which recognizes SUMO1 and SUMO2/3 (Figure 42). The higher molecular weight bands observed indicate that TRF2 is SUMOylated in MCF7 cells (Figure 42).

Summary

SUMOylation of TRF2 has been identified in ALT cells, along with the SUMOylation of TRF1, TIN2 and Rap1 (Potts and Yu, 2007). However, in telomerase positive or normal cells, this modification has yet to be recognized. Possible SUMOylation sites were predicted and SUMOylation of TRF2 was verified both *in vitro* and in telomerase positive MCF7 cells to determine the function of its SUMOylation. Mutation of two of the predicted sites, those with the highest probability of being true SUMO binding sites at K140 and K245, resulted in only a modest decrease in SUMOylation *in vitro* when combined.

DNTRF2 appeared to not be SUMOylated. The two SUMO sites mutated in these experiments remained within the DNTRF2 coding sequence and because the DNTRF2 truncation was not SUMOylated, a third site located outside of the DNTRF2 sequence (at K459) could play a major role in DNTRF2's effects in telomerase-positive cells. DNTRF2, when overexpressed in cells, results in end-end fusions and genomic instability, eventually leading to cellular senescence or death (van Steensel, *et al.*, 1998; Karlseder, *et al.*, 1999). The elimination of the SUMO moiety at the K459 binding site could be a factor in this damage response. Mutation of K459 will be performed to determine if this third site is an important SUMO binding site for proper function of TRF2.

SUMOylation, unlike ubiquitination, has a variety of functions in the cell, including localization, degradation, and protein-protein interactions. It is possible that SUMOylation of

TRF2 functions in maintaining TRF2's interactions with other telomere proteins, although an examination of TRF2/Rap1 or TRF2/TIN2 interactions using the SUMO-deficient mutant of TRF2 would need to be performed. PARP1, a TRF2 interacting protein that functions to poly(ADP)-ribosylate TRF2, and DNA-PKcs, a protein kinase that binds TRF2, may be affected by the inhibition of TRF2 SUMOylation, possibly repressing their role in post-translation modification (reviewed in: De Boeck, *et al.*, 2009). Thus, SUMOylation may be having an effect on other post-translational modifications of TRF2.

The importance of TRF2 in maintaining and protecting the telomere requires a complete understanding of its regulation and post-translation modifications. Little is known in the literature about modifications of TRF2 in telomerase-expressing tumor cells. We have identified a novel post-translational modification of TRF2 by SUMOylation in telomerase-positive cancer cells. In an effort to determine the functional significance of this modification, the various TRF2 SUMO-mutants we have created may lead to elucidation of a role for SUMO in maintaining telomere protein-protein interactions, localization, or degradation.

Vita

Amy Nicole Depcrynski was born November 20, 1979, in Richmond, Virginia. She graduated from The Governor's School for Government and International Studies, Richmond, Virginia in 1998. She received her Bachelor of Science in Biology with a minor degree in Chemistry from Virginia Commonwealth University in 2003 and joined the Medical College of Virginia at VCU in 2004.

Publications

Kmieciak M, Worschech A, Gowda M, Heidari N, Wang E, **Depcrynski A**, Knutson KL, Holt SE, Marincola FM, and Manjili MH. 2009. Tumor-specific effector and memory CD8+ T cells can be generated and maintained in the absence of CD4+ T cells capable of rejecting recall tumor challenge. Submitted.

Depcrynski, A.N., P.C. Sachs, L.W. Elmore, and S.E. Holt. Regulation of Telomerase Through Transcriptional and Post-Translational Mechanisms, in "Telomeres and Telomerase in Cancer". (2009). Springer, Cancer Drug Discovery and Development series. Editor: Keiko Hiyama.

Strub, G.M., **A.N. Depcrynski**, L.W. Elmore, and S.E. Holt. 2008. Recovery from Stress is a Function of Age and Telomere Length. *Cell Stress and Chaperones* (2008) 13:475-482.

Honors and Awards

2008-2009	Who's Who Among Students in American Universities and Colleges
2007	Phi Kappa Phi Honors Society
2007-2008	Who's Who Among Students in American Universities and Colleges
2005-2006	Charles C. Clayton Award for Scholarly Achievement
2004	Human Genetics Excellence Award, Department of Human Genetics, Virginia Commonwealth University School of Medicine, Richmond, VA

Teaching Experience

2008-2009	Supervisor: Emily Rice, VCU undergraduate project "Expression of an Inducible Human Telomerase Gene in a Normal Fibroblast Cell Line" BIOL 451/Biology of Cancer part II
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- 2008-2009 Supervisor: Patrice Mann, VCU undergraduate project
“Radicicol Treatment in H1299 Cells, Effects on Cell Growth and Chaperone Levels” BIOL 451/Biology of Cancer part II
- 2007-2009 Supervisor: Rebecca Breed, Collegiate High School
Senior Thesis Project “Human Telomerase expression in Fish Cell Lines”
- 2006-2007 Supervisor: Anne Ruskin, VCU undergraduate project
“Sumoylation in Cancer Cells” BIOL 451/Biology of Cancer part II
- 2005-2007 M1 Medical Student Group Recitation, Virginia Commonwealth University, Medical Campus, Richmond, VA
- 2005-2007 Life Science 101 Recitation, College of Humanities and Science, Virginia Commonwealth University, Richmond, VA