Introduction

TCTP, also known as Fortilin/Histamine Releasing Factor (HRF), was first discovered over two decades ago as a growth promoting factor in Ehrlich ascites tumor [1]. Since then, a diverse range of biological functions have been attributed to the protein including essential roles in cell proliferation and growth regulation [2,3,4,5], histamine releasing properties and other ‘cytokine-like’ activity [6,7,8,9] and antiapoptotic activity. TCTP is overexpressed in many human cancers including prostate, liver and breast [10,11,12] and tumour reversion results in its downregulation [4]. TCTP's anti-apoptotic function is attributed in part to interactions with both anti-apoptotic (Mcl-1 and Bcl-xl) [13,14] and pro-apoptotic (BAX) [15] members of the Bcl-2 family. Additionally, TCTP has been ascribed a role in DNA damage sensing and repair, forming complexes with ATM and the DNA binding subunits Ku70 and Ku80 of DNA-dependent protein kinase [16]. More recently, TCTP has been shown to bind directly to p53, with TCTP overexpression increasing p53 degradation and promoting lung cancer cell survival [17]. Amson et al have recently demonstrated binding between TCTP and the E3 ubiquitin ligase HDM2 [18]. This interaction appears to control p53 levels by inhibiting HDM2 auto-ubiquitination, thereby promoting p53 ubiquitination and degradation.

In this study, we mapped the TCTP binding site to the N-terminal, p53-binding domain of HDM2, and found that mutations in the HDM2 α2 helix forming part of the p53 binding cleft significantly compromise binding. The HDM2 binding site on TCTP was also mapped to the basic domain 2 of TCTP, with residues 80–133 playing a crucial role in the interaction.

Nutlin-3 is a small molecule which binds to the p53 binding pocket of HDM2, thereby inhibiting wild type p53-HDM2 interaction, attenuating p53 degradation and activating cell cycle arrest/apoptosis mediated by the p53 network [19]. We further demonstrate that Nutlin-3 inhibits the TCTP-HDM2 interaction both in vitro and ex vivo, thus highlighting an additional mechanism through which Nutlin-3 abrogates HDM2 function.

Materials and Methods

Reagents

DO1 antibody was a kind gift from Dr Borivoj Vojtesek. Anti-FLAG and anti-HA antibodies were from Sigma. Nutlin-3 was from Calbiochem. The following oligonucleotides (FBCO) were used:
1) TCTP-F: 5'-ATGATTATCTACC GGAGCTC-3'
2) TCTP-R: //5'-TTAACATTTTTTCCATTTCTAAAC-3'
3) TCTPINF-F: 5'- AAGGAGATATACATATGATTATCA-3'
4) TCTPFLAGINFR: 5'-GGTGTTGTGCTTGGAGTATTTGATCATCATGCTTATTAATCA-3'
5) TCTPFLAGINFR: 5'- CTCCATTTTCCATTTCTAAACATCC -3'
In vitro transcription-translation

Proteins were synthesised by in vitro transcription/translation using the PURESYSTEM kit (NEB). 10 ng of HDM2 PCR template (~1.7 Kb) was used per 5 μL reaction. The amounts of all other templates were appropriately adjusted to maintain same molar concentration. ZnCl2 was added to a final concentration of 0.5 μM for expression of HDM2 and p53 proteins. p53 protein was synthesised at 37°C for 0.5 hours. All other proteins were synthesised at 37°C for 1 hour. Completed reactions were incubated on ice until required.

Pull-down assays

Protein G beads (Invitrogen) were incubated with anti-HA antibody or anti-Flag antibody (1 μg per 5 μL beads) for 1 hour in PBS+1% (w/v) BSA and subsequently washed twice in PBS+0.1% (w/v) BSA and once in PBS to remove non-specifically bound protein. In vitro synthesised protein (5 μL per 5 μL beads) was added directly to beads and incubated on a rotator for 45 minutes. Beads were washed and incubated in vitro extract containing second protein as before. For competition experiments, beads were incubated with Nutlin-3, p53 peptide/control peptide [20] in PBS for 45 minutes before washing and addition of second protein. Beads were finally washed as before and bound proteins eluted by resuspension in 20 μL SDS-PAGE loading buffer and incubation at 95°C for 5 minutes. Where required, blank in vitro extract (no template DNA added) was used as control.

For pull-downs using peptides the following bionitilised peptides (Mimotopes) were used:

TH2: GGGSTSFTKＥ AYKVKYKDМKSКGКL ЕCPR

TH3: GGGSRPERVKPMTGAAEQI KHIЛАНКЯНУQ

TH3-NL: GGGVКPFМTAGЕСQHKИЛАНКЯНУQ

TH3-L: GGGSAKDTYTМKЕLФLЯQIМTMK

GS-control : GGGSGGGGSGGGGSGGGGSGGGG

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Protein purification

Both TCTP and HDM2 (amino acids 1–125) were expressed as GST-fusion proteins using the pGEX-6P-1 expression vector. Both proteins were initially passed through a 5 mL GSTrap™ FF (GE life sciences) column and eluted following on-column cleavage with precission protease. Protein fractions were analyzed with SDS page gel and concentrated using a Centricon (3.5 kDa MWCO) concentrator (Millipore). HDM2 protein samples were then dialyzed into a buffer solution containing 20 mM Bis-Tris, pH 6.5, 0.05 M NaCl with 1 mM DTT and loaded onto a monoS column pre-equilibrated in buffer A (20 mM Bis-Tris, pH 6.5, 1 mM DTT). Bound protein was eluted with a linear gradient of 1 M NaCl over 25 column volumes. For TCTP the same protocol was followed but buffers instead contained 20 mM Tris at pH 8.0 and the protein was loaded onto a monoQ column before being eluted. Protein fractions were identified using SDS page gel and protein concentration measured using absorbance at A280.

Figure 1. TCTP interacts with the N-terminal region of HDM2. A, in vitro pull-down of TCTP by C-terminally truncated HDM2 variants. Upper panel, Western blot of pulled down TCTP (anti-FLAG antibody). Lower panel, input levels of respective HDM2 variants (anti-HA antibody, arrowed). B, in vitro pull-down of TCTP by HDM2 (110–491). Upper panel, Western blot of pulled down TCTP. Lower panel, input levels of respective HDM2 variants (arrowed). C, in vitro pull-down by HDM2 N-terminal domain deletion mutants. Upper panel, Western blot of pulled down TCTP. Lower panel, input levels of respective HDM2 variants (arrowed). Control lanes indicate TCTP pull-down in the absence of HDM2. D, in vitro pull-down of TCTP by HDM2 with point mutations in α2 helix. Upper panel, Western blot of pulled down TCTP (anti-FLAG antibody) by indicated mutants. Lower panel, input levels of respective HDM2 proteins.

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Figure 2. Inhibition of TCTP-HDM2 interaction by Nutlin-3 and p53 peptide. A, in vitro translated HDM2 was immobilised on beads and pre-treated with indicated amounts of Nutlin-3 or p53 peptide/p53 control peptide (1 mM). Bound TCTP detected by Western blot (anti-FLAG antibody). Control lane indicates TCTP pull-down in the absence of HDM2. B, Recombinant HDM2 (residues 17–125) or control peptide (GS-control) was immobilised on beads and incubated with recombinant TCTP either in presence or absence of 200 μM Nutlin-3. doi:10.1371/journal.pone.0042642.g002

Figure 3. Nutlin-3 does not inhibit p53 binding to HDM2-M62A. In vitro translated HDM2 or HDM2-M62A was immobilised on beads and pre-treated with 0/100/200/400 μM Nutlin-3 prior to incubation with in vitro translated p53. Bound p53 detected by Western blot using DO1 anti-p53 antibody. Control lane indicates p53 pull-down in the absence of HDM2.

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Western blot Analysis

Immunoprecipitated proteins were subjected to electrophoresis, transferred to nitrocellulose membranes and probed for TCTP with horseradish peroxidase conjugated anti-flag antibody (Sigma) or for HDM2 with anti-HA antibody followed by rabbit anti-mouse (Dakocytomation). P33 was probed for using horseradish peroxidase conjugated DO1 antibody (Santa Cruz). For peptide-pull-down assays, TCTP was detected with anti-TCTP antibody (ab37506, Abcam) and HDM2 was detected with 4B2a anti-HDM2 antibody.

Fluorescence polarization (FP)

Fluorescence polarization measurements were performed using purified HDM2 (1–125) protein and carboxyfluorescein (FAM) labeled 12-1 peptide (FAM-RFMDYWEGL-NH2) on the En-Vision™ Plate Reader (Perkin Elmer). Competition measurements were carried out in triplicate, containing 50 nM of fluorescence peptide, with or without 250 nM of HDM2 (1–125) and the respective competitors (TH2, TH3, Nutlin-3, p53 peptide or GS-control peptide) in 50 μL of PBS-0.005%(v/v)Tween-20 buffer.

Cell culture

H1299 p53−/− cells [21] were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin. The cells were seeded at 1.4×10^5 cells/well in 6-well plates, 24 hours prior to transfection. A total of 1.375 μg of expression construct DNA was transfected per well with lipofectamin (Invitrogen) according to the manufacturer’s instructions. MG132 (Calbiochem) was also added at a final concentration of 20 μM 4.5 hours post-transfection to prevent proteasomal degradation. 10 μM Nutlin-3 was added to selected wells 4.5 hours post-transfection to prevent proteasomal degradation. 10 μM Nutlin-3 was added to selected dishes 24 hours post seeding. Drug treatment was allowed to proceed for 1 hour prior to harvesting.

Immunoprecipitation and western blot analysis

H1299 p53−/− cells were harvested 24 hours after transfection and lysed with lysis solution (Applied Biosystems) supplemented with both protease and phosphatase inhibitors. 10 μL of anti-HA (Sigma) antibody-coated protein G Dynabeads (Invitrogen) was used per reaction. Beads were washed twice in PBS with 0.1% (v/v) Tween-20 and incubated with 150 μg of cell lysate on a rotator at 4°C for 3 hours before washing three times with PBS with 0.1% (v/v) Tween-20. The beads were resuspended in 20 μL of SDS-PAGE loading buffer and the protein complexes eluted by incubation at 95°C for 5 mins. HCT116 p53−/− cells were harvested 1 hour post drug treatment and lysed with modified RIPA buffer (50 mM Tris-HCl pH 7.4–8.0, 150 mM NaCl, 1% NP-40). Beads were prepared as above and incubated with 1 μg cell lysate at 4°C overnight with 2 μg of 2A9 antibody (Abcam). The beads were then washed as described for H1299 p53−/− cells and the protein complexes eluted by incubation at 95°C for 5 mins. Immunoblotting was carried out with the relevant antibodies and identified by Immun-star™ westernCTM kit (Bio-rad). 5 μg of H1299 p53−/− cell lysate and 20 μg of HCT116 p53−/− cell lysate per reaction was also used to check expression levels of relevant proteins via western blot.

Results

We first carried out pull-down assays using in vitro expressed proteins to map the interaction site(s) between TCTP and HDM2. HDM2 (tagged at the C terminus with HA) was bound to protein G beads coated with anti-HA antibody. The beads were subsequently incubated with TCTP (FLAG-tagged). Bound TCTP was identified via Western blot. C-terminal deletion analysis of HDM2 indicated the N-terminal region alone (residues 1–83) was sufficient for interaction with TCTP (Figure 1A). Notably, when compared to full-length HDM2, deletion of C-terminal residues 303–491 (containing the zinc finger and RING domains) and 340–491 (containing the RING domain) led to increased interaction with TCTP. Deletion of residues 1–109 in HDM2 resulted in very minimal interaction with TCTP. Deletion of residues 1–109 in HDM2 led to increased interaction with TCTP (Figure 1B), suggesting a predominant N-terminal interaction site. Further deletion analysis highlighted the importance of residues 44–65 in the interaction (Figure 1C). This region comprises the α2 helix that forms part of the p53 binding cleft of HDM2 [23]. Alanine scanning of this region was carried out to further map the interaction. Residues Y48, L54, Y56, Y60 and M62 were individually mutated to alanine in full length HDM2 and the interaction with TCTP assayed. The results show a progressive reduction in the
interaction with TCTP as residues along the α2 helix are mutated, with the M62A mutant showing considerably weaker binding (Figure 1D). We additionally deleted the first 25 amino acids in HDM2 comprising the flexible lid region [24]. Binding to TCTP was unperturbed, further confirming the importance of residues 44–65 in the interaction with TCTP (Figure 1D). M62 forms part of the binding pocket that accommodates the side chain of F19 in the p53 transactivation domain [25]. We therefore investigated whether the TCTP binding site of HDM2 overlapped with the p53 binding site. HDM2 was first incubated with a p53 peptide corresponding to residues 19–26 of the p53 transactivation domain that interact with HDM2 [25], followed by TCTP. We also pre-incubated with the HDM2 inhibitor Nutlin-3, which binds to the p53-binding cleft [19]. p53 peptide, but not control peptide (p53 peptide with critical contact residues F19, W23, L26 mutated to alanine) diminished TCTP binding (Figure 2A). Nutlin-3 also showed a dose responsive reduction in TCTP binding. Inhibition by Nutlin-3 was again observed when recombinant HDM2 N-terminal domain was used to pull down recombinant TCTP (Figure 2B).

We further investigated the HDM2-M62A mutant to see if it retained the capacity to bind p53. As shown in Figure 3, it bound to p53 as strongly as wild-type, indicating the lack of any major structural perturbation due to this mutation. However, whilst Nutlin-3 showed a dose responsive knock down in the HDM2-p53 interaction, the M62A mutant proved recalcitrant to Nutlin-3 inhibition.

We additionally carried out C-terminal deletion analysis of TCTP to map its interaction site with HDM2. Whilst full-length TCTP and residues 1–133 bind to HDM2, further truncation to 79 residues completely ablates HDM2 binding (Figure 4A). The same result was obtained with the reverse configuration of the IP (TCTP captured on beads used to pull down HDM2, Figure 4B). This indicated a probable interaction interface within amino acids 80–135 of TCTP which comprises an helix-loop-helix motif. We
next carried out a series of pull-down experiments using 2 synthetic peptides spanning this region of TCTP (TH2: residues 81–110; TH3: residues 107–133) (Figure 5A) along with a peptide spanning the HDM2 α2 helix (residues 43–65). The results in Figure 5B (top panel) indicate that both TH2 and TH3 peptides immobilised on beads can pull down recombinant HDM2 N-terminal domain (residues 1–125), with TH3 showing a stronger binding phenotype. Additionally, immobilised HDM2 α2 helix peptide pulled down recombinant full-length TCTP (bottom left panel). Strikingly, the same peptide with the M62A mutation (α2M62A) showed significantly reduced pull-down of TCTP. Neither recombinant TCTP nor HDM2 (1–125) bound to an immobilised control peptide (CON, top panel). As a positive control we used the p53 peptide known to interact with the N-terminus of HDM2, with TH3 showing a stronger binding phenotype. This was also observed when the interactions were assayed by fluorescence polarisation (Figure 5D). HDM2 (1–125) was pre-incubated with fluorescently labelled p53 peptide and the ability of the TCTP peptides to displace this was measured. TH3 peptide, but not TH2 was able to displace the p53 peptide, although to a lower extent than the positive controls Nutlin-3 and un-labelled p53 peptide.

We next investigated the effect of Nutlin-3 on the endogenous TCTP-HDM2 interaction in the HCT116 p53−/− cell line. Co-immunoprecipitation was carried out using anti-HDM2 antibody to capture TCTP-HDM2 complexes. The results (Figure 6A) indicate disruption of the TCTP-HDM2 interaction by Nutlin-3, consistent with the previous in vitro data (Figures 2, 5C). The same phenotype was seen using exogenously expressed HDM2 in the p53-null H1299 cell line (Figure 6B). Furthermore, the HDM2-M62A mutant showed very weak interaction with TCTP compared to wild-type, again consistent with the in vitro result (Figure 1D).

**Discussion**

It was recently shown that TCTP increased MDM2-mediated ubiquitination of p53, and that this effect was inhibited by Nutlin-3 [18]. In the present study, we provide a possible mechanistic rationale for this observation by showing that TCTP and Nutlin-3 can compete for binding to the p53-binding cleft in the N-terminus of HDM2. The p53-binding cleft consists of 4 α helices and a pair of β sheets cap each end [25]. Deletion analysis implicated the α2 helix forming one side of the cleft as contributing significantly to the TCTP interaction site. Alanine scanning of the α2 helix further identified critical residues involved in the interaction, with M62 being of particular importance. This residue comprises part of the binding pocket that accommodates F19 of p53 and the ethyl ether moiety of Nutlin-3 [19]. Notably, binding of p53 to HDM2-M62A was not inhibited by Nutlin-3, suggesting against mutation-induced structural deformation. Based on these observations, we propose a model wherein TCTP binds a sub-region of the p53-binding cleft to exert its chaperone-like function on HDM2. TCTP is subsequently displaced by p53 due to its higher affinity for the p53-binding cleft. Additionally, a secondary p53 interaction site within the acidic domain of HDM2 [26,27] may contribute towards high affinity interaction and TCTP displacement.

A highly allosteric model of HDM2 function has emerged, wherein conformational changes within structurally discrete domains impact on its interaction with p53 [28]. Notably, the C-terminal RING finger domain (residues 438 to 479) regulates the binding affinity of the N-terminal region to p53, and mutations in this domain have also been shown to modulate Nutlin-3 efficacy [29]. Our results indicate increased interaction of HDM2 with TCTP when the C-terminal zinc finger (residues 300 to 332) and/or RING domains were deleted. Allosteric modulation by these domains may therefore also regulate the HDM2-TCTP interaction.

We additionally mapped the TCTP interaction site to within residues 80–133 corresponding to the basic domain 2. This region comprises an helix-loop-helix motif [30] and our data show residues within the loop to contribute significantly to the
interaction with HDM2. Domain 2 has been implicated in TCTP’s interaction with tubulin [2], calcium [31], and the Na,K-ATPase α subunit [32]. Furthermore, TCTP has recently been shown to interact with p53 through either domain 2 [17] or N- and C-terminal regions [33]. We note that Amsen et al. have mapped an interaction interface between residues 1–68 of TCTP and residues 302–435 of HDM2 using SPR and recombinant proteins [18]. This interaction site was not evident in our results using pull-down assays with in vitro expressed proteins. We are presently carrying out further work to evaluate the contribution of this additional binding interface to the overall TCTP-HDM2 interaction both in vitro and ex vivo.

Using molecular simulations, a docked complex of TCTP with HDM2 (1–125) was derived (Figure 7). Stable interactions of the TH2 and TH3 helices of TCTP with residues in the HDM2 nutlin-binding pocket were observed in accordance with the alanine scanning data (Figure 1D). The RPER loop region (residues 107–110) connecting TH2 and TH3 is stabilized by intramolecular interactions of R107 and R110 with residues in TCTP, whilst the backbone carbonyl of P108 and the side chain of E109 are stabilized by K51 of HDM2. Additionally, E104 of TCTP is also stabilized by K45 of MDM2 (Figure 7A). The loss of affinity seen when the loop region was deleted from peptide TH3 (Figure 3B) could result from removal of one salt bridge and/or significant perturbation of the other. It is clear from Figure 7A that the α2 helix of HDM2 interacts with both TH2 and TH3 of TCTP, with M62 closely packed under TH3.

TCTP has been shown to be significantly upregulated in a number of human cancers, with high levels of TCTP correlating with poor prognosis [10,11] [12,18]. Nutlin-3 has been shown to be most effective in cancers which express wild type p53 and high levels of HDM2. Investigation of the effects of Nutlin-3 in cancer cells and animal models with high levels of TCTP overexpression may prove valuable.

The value of inhibiting the p53-HDM2 interaction as a possible target for cancer therapeutics is currently an area of great activity. The discovery that TCTP not only interacts with both these proteins, but has a binding site on HDM2 which overlaps with that of p53, adds further complexity to the p53-HDM2 interaction model. Further work must be done to understand the cellular implications of these interactions and their consequences for therapies aimed at inhibiting p53-HDM2 binding.

Author Contributions
Conceived and designed the experiments: DL, FG. Performed the experiments: GF WG SJW QST LJT. Analyzed the data: CB DL CV. Contributed reagents/materials/analysis tools: QST CB. Wrote the paper: GF FG.

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