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Role of p53 in cAMP/PKA pathway mediated apoptosis

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Abstract

Deregulated β -adrenoceptor/cAMP-PKA pathway is implicated in a range of human diseases, such as neuronal loss during aging, cardiomyopathy and septic shock. The molecular mechanism of this process is, however, only poorly understood. We recently had demonstrated that the β -adrenoceptor/cAMP-PKA pathway triggers apoptosis through the transcriptional induction of the pro-apoptotic BH3-only Bcl-2 family member *BIM* in tissues, such as the thymus and the heart. Induction of *BIM* is driven by the transcriptional co-activator CBP (CREB Binding Protein) together with the proto-oncogene c-Myc. Association of CBP with c-Myc leads to altered histone acetylation and methylation pattern at the *BIM* promoter site [1]. However since CBP is a co-factor for multiple transcription factors, BH-3 only proteins other than Bim could also contribute to this apoptosis pathway. Here we provide evidence for the involvement of p53-CBP axis in apoptosis through Puma/Noxa induction, in response to β -adrenoceptor activation. Our findings highlight the molecular complexity of pathophysiology associated with a deregulated neuro-endocrine system and for developing novel therapeutic strategies for these diseases.

Keywords: Apoptosis, beta adrenergic pathway, Bim, Puma, p53, CBP.

Introduction:

Cyclic adenosine monophosphate (cAMP) is a second messenger conserved throughout evolution. In bacteria, it regulates catabolite repression and quorum sensing [2,3] whereas in lower forms of eukaryotes such as *Dictyostelium*, it regulates chemotaxis and differentiation [4]. In metazoans, its primary role is to act as an intracellular carrier of metabolic information, for example regulating hormonal responses [5] and in eliciting apoptotic responses and regulating ontogeny [6,7]. cAMP-regulated Protein Kinase A (PKA) is the main target of cAMP flux in mammals. Using transgenic mouse models, it is now firmly established that deregulated cAMP/PKA pathway leads to apoptosis [8,9].

Aging and chronic stress in humans is associated with marked and sustained increases in the sympathetic nervous system activity in several peripheral tissues, including the heart and thymus [10]. This increased activity produces excess catecholamines, which bind β ARs and induce cAMP production [11]. This catecholamine-induced beta-adrenergic receptor (β AR) activation or by autoantibodies as seen in Grave's disease [12] could lead to PKA-mediated cardiomyocyte apoptosis resulting in heart failure [13]. β ARs also exist on the surface of both B and T cells which are known to affect migration and activation of these lymphocytes [14]. Catecholamines appear to regulate the activity of immunocompetent cells and this could partly explain the immunological privilege status of the central nervous system. In addition, catecholamines such as adrenaline are used in the acute phase of septic shock to improve hemodynamic status of the heart [15] and this is associated with profound alterations in immune cells due to apoptosis [16]. Catecholamine mediated apoptosis is also attributed to the loss of dopamine receptor containing neurons during aging [17] and to stress-induced immune modulation [18].

The molecular basis of β AR-cAMP/PKA axis in inducing apoptosis is yet to be determined. The BH3-only Bcl-2 family protein Bim is considered to be an essential initiator of apoptosis in a wide variety of physiological settings including calcium flux, growth factor withdrawal [19], endoplasmic reticulum stress [20], T cell receptor activation [21] and B cell receptor activation [22]. We had previously reported that the cAMP/PKA pathway has a significant effect on Bim at the protein level [23]. The major isoform of Bim (BimEL) interacts with the regulatory subunit of PKA, PRKAR1A. This complex acts as scaffolding structure that brings the catalytic subunit of PKA (PKAC α) in close proximity to BimEL resulting in its phosphorylation. Phosphorylation of Bim by PKA enhances the

stability of the protein. Mutations that abolish the phosphorylation result in the rapid turn over of Bim and reduced apoptotic ability.

In S49-T lymphoma cell line, it was shown that Bim was essential for cAMP/PKA activated cell death [24]. The transcriptional induction of *BIM* was attributed to the transcription factors ATF1 and CREB based on the presence of a cAMP Responsive Element (CRE) on the *BIM* promoter [25]. However, a direct evidence for ATF1/CREB engagement to the *BIM* promoter is still lacking. We recently had shown that the transcription factor c-Myc and the co-factor CREB binding protein (CBP) are necessary for cAMP/PKA-induced transcriptional regulation of *BIM* [1]. We also had shown that in cardiomyocytes and in thymocytes, cAMP/PKA-induced apoptosis is Bim dependent. However, these results do not exclude the possibility that other BH3-only proteins may also contribute to this apoptosis pathway. In the present work, we show that *PUMA*, another BH3-only Bcl-2 family member plays a significant role in regulating cAMP/PKA-induced apoptotic pathway. p53 plays an important role in the transcriptional regulation of *PUMA* and we provide evidence that during cAMP/PKA activation, the transcriptional mechanism for *PUMA* and another BH3-only gene *NOXA*, involves an interplay between CREB binding protein (CBP) and p53.

Materials and Methods

Cell culture, Transfection, Lentiviral/Retroviral Infection

Mouse embryonic fibroblasts (MEFs) and LK-2 cells were cultured in DMEM supplemented with 10% fetal calf serum (Invitrogen, Carsbad, USA) at 37°C in a humidified 10% CO₂ incubator. Wild type and various knock-out MEFs were generated from E15 embryos in accordance with standard procedures and were infected with SV40 large T antigen expressing lentivirus. To generate lentiviral particles, 293T cells were transfected with packaging constructs pCMV δ R8.2 and VSVg and the relevant lentiviral plasmid at a ratio of 1:0.4:0.6 using Fugene 6.0TM transfection reagent (Promega corporation, Fitchburg, USA) following the manufacturer's instructions. The virus containing supernatants were harvested, filtered (0.8 μ M), and supplemented with Polybrene (4 μ g/mL). Target cells were infected with virus supernatant as described by Lee et al, [1].

For retroviral infection, pMSCV based vectors carrying gene of interest were transfected into Phoenix cells using using Fugene 6.0TM transfection reagent (Promega Corporation, Fitchburg, USA)

following the manufacturer's instructions. The virus containing supernatants were harvested, filtered (0.8 μ M), and supplemented with Polybrene (4 μ g/mL). Target cells were infected with virus supernatant as described by Gangoda et al, [26]. GFP positive cells were sorted by FACS Aria III cell sorter (Becton and Dickinson, New Jersey, USA).

Cell death analysis

Cells were seeded at density of 5×10^4 per well in a 12-well plate and were allowed to adhere for 24 hours at 37°C and 10% CO₂. These cells were treated with 10 μ M dobutamin (Cat# D0676 Sigma, Castle Hill, NSW, Australia) and 10 μ M rolipram (Cat# R6520 Sigma, Castle Hill, NSW, Australia) in serum free DMEM medium for 24 and 48 hours. The cells were then centrifuged at 1500 rpm for 5 minutes and the pellet was resuspended in 100 μ l of Annexin-V FITC (1:1000 in KDSBSS buffer (1.68M KCl, 1.12M CaCl₂, 1.68M MgSO₄, and 1.68 M KH₂PO₄). Following incubation on ice for 30 minutes the cells were washed with 3.5ml KDSBSS buffer and centrifuged at 151g for 5 minutes, supernatant was discarded and the cells were vortexed briefly followed by addition of 100 μ l of propidium iodide (1.2 μ g/ml). Samples were analysed by flow cytometry using a FACS-Calibur (BD Biosciences, NJ, USA).

Quantitative PCR

Total RNA from MEFS was isolated using TRIZOL®. Complementary DNA (cDNA) was synthesized from 2 μ g of total RNA using the Superscript III RT-PCR system (Cat# 18080-051 Invitrogen, Carlsbad, USA), using oligo-dT primer according to manufacturers instructions. qPCR was carried out using Brilliant II SYBER Green QPCR master Mix (Cat# 600828 Stratagene, La Jolla, USA). All cDNA samples were tested in triplicate using a Light cycler® 480 Real-time PCR instrument (Roche Applied Science, Penzburg, Germany). Following primers were used for qPCR reactions: *BIM* (F): GAGTTGTGACAAGTCAACACAAACC; *BIM* (R): GAAGATAAAGCGTAACAGTTGTAAGATA; *NUR77* (F): CCTGTTGCTAGAGTCTGCCTTC; *NUR77* (R): CAATCCAATCACCAAAGCCACG; *PUMA* (F): ATGCCTGCCTCACCTTCATCT; *PUMA* (R): AGCACAGGATTCACAGTCTGGA; *NOXA* (F): ACTGTGGTTCTGGCGCAGAT; *NOXA* (R): TTGAGCACACTCGTCCTTCAA

Western blot analyses

Western blot analyses were performed as described before [23]. The following antibodies were used: Anti Bim (rat monoclonal 3C5, kind gift from Prof. Andreas Strasser), anti Puma (3043-P, rabbit polyclonal, ProSci, San Diego, CA, USA), anti-p53 (NCL-p53-CM5p, rabbit polyclonal, Leica

microsystems, Newcastle, UK), anti-p21 (ab18209, rabbit polyclonal, Abcam, Cambridge, UK), and anti-HSP70 (JG-1, MA3-028, Thermo Scientific, Scoresby Vic, Australia).

Results:

***PUMA* co-operates with *BIM* in inducing β AR-mediated apoptosis**

We had recently demonstrated that in breast cancer epithelial cells (MCF7), primary thymocytes and cardiomyocytes, Bim plays a crucial role in initiating apoptosis in response to β AR activation. The mechanisms involved post-translational stabilization of Bim protein by PKA phosphorylation [23] or the transcriptional induction mediated through the CBP/c-Myc axis, which leads to epigenetic changes at the *BIM* locus [1]. However, in mouse embryonic fibroblasts (MEFs), though β AR signalling induces *BIM*, *BIM* ablation does not protect against β AR signalling. This suggested that additional BH3-only proteins play a role in this death pathway. Therefore we used a variety of MEFs, i.e. *BIM*^{-/-}, *BIM*^{-/-} *PUMA*^{-/-} DKO, *TP53*^{-/-}, *BAX*^{-/-} *BAK*^{-/-} DKO MEFs to study their viability following β AR activation. These MEFs were treated with a pan- β AR agonist (Dobutamine [27]) and the phosphodiesterase inhibitor rolipram. The percentage of viable cells 24 and 48 hours after β AR activation clearly indicates that while *BIM* and *PUMA* ablation individually did not offer any protection from apoptosis, their combined ablation resulted in a significant protection. This protection was also seen in p53 knockout cells (Fig. 1A). Since deletion of BAX and BAK protected the cells almost completely from apoptosis, these data show that the intrinsic mitochondrial apoptotic pathway exclusively regulates this death in a manner dependent on both Bim and Puma and possibly other p53-dependent proteins such as Noxa, and requiring the activation of Bax and Bak. Consistent with this observation, we could see a robust induction of both Bim and Puma in MEFs treated with the pan- β AR agonist Dobutamine (Fig. 1B).

Transcriptional induction of pro apoptotic genes during cAMP/PKA activation

Having established that combined ablation of *BIM* and *PUMA* leads to protection from apoptosis, we next examined the transcriptional induction of various pro-apoptotic genes in response to β AR stimulation. Consistent with the apoptotic response, we observed rapid and robust induction of both *BIM* and *PUMA* during β AR stimulation. Since p53 ablation offered similar protection to combined *BIM* and *PUMA* deletion, we reasoned that other p53 target genes could also be induced. We measured the expression of *NOXA*, a p53-induced BH3-only protein and observed induction in response to β AR stimulation (Fig. 2A). *NUR77* expression was used as a control, since this gene is also induced by β AR

stimulation but is not p53-dependent [28]. Importantly, induced *PUMA* and *NOXA* expression was not seen in *TP53*^{-/-} MEFs, whereas *NUR77* remained β AR-inducible (Fig. 2B). These results show that in MEFs, β AR stimulation leads to transcriptional induction of p53 target genes such as *PUMA* and *NOXA*.

Role of p53 during β AR activation

Adrenaline has been reported to lead to β -arrestin-mediated regulation of p53 through the activation of β AR-PKA pathway in the thymus [29]. However, the stress response we observed in the thymus is different from MEFs as *BIM* ablation alone offered complete protection from apoptosis in response to β AR activation in thymus but not in MEFs [1]. Since *TP53* deletion offered protection against apoptosis and resulted in reduced transcription of p53 target genes such as *PUMA* and *NOXA*, we wanted to further elucidate the role of p53 in β AR-mediated apoptosis. We used a GFP reporter with upstream p53 binding sites (cloned from the *PUMA* promoter) [30]. Infection of wild type MEFs with the reporter resulted in a strong expression of the GFP reporter in contrast to infection of *TP53*^{-/-} MEFs, which had substantially reduced baseline levels of GFP expression (Fig 3A). This confirmed that the GFP expression from this reporter was p53-dependent (Fig. 3A). When these MEFs were treated with β AR agonist, dobutamine, there was a significant increase in the level of GFP expression in the wild type MEFs and this response was muted in *TP53*^{-/-} MEFs suggesting that p53 is activated in response to β AR activation (Fig. 3B). This finding was corroborated by analysing the expression levels of p21, a downstream target of p53. While wild type MEFs induced p21 protein level during β AR activation, this effect was not observed in *TP53*^{-/-} MEFs (Fig. 3C). It has been reported that β AR activation leads to the down regulation of p53 protein levels in thymocytes [29]. However, this mode of regulation did not appear to be playing any role in p53 regulation in MEFs in response to β AR activation. If this were the case, one would expect the levels of *PUMA* to be down regulated as well. Indeed, Western blot analysis clearly showed that the steady state levels of p53 remained the same (Fig. 3D).

Role of CBP in p53-mediated *PUMA* induction

We had previously shown that the transcriptional co-factor CBP (CREB-Binding Protein) was necessary for c-Myc mediated *BIM* up regulation during β AR activation [23]. CBP is a histone acetyl transferase that associates with a variety of transcription factors including p53 and FoxO3a [31,32]. Therefore, we wanted to see if CBP was involved in regulating *PUMA* expression. To this end we used the lung carcinoma cell line LK-2, which does not have a functional CBP protein owing to a deletion of

exon 3 of the *CBP* gene [33]. We expressed functional FLAG-tagged CBP gene in these cells using 4-hydroxytamoxifen (4-OHT)-inducible lentiviral expression system (Fig. 4, inset). While the wild type LK-2 cells failed to induce *PUMA* in response to β AR activation, the LK-2 cells with functional CBP was able to induce *PUMA* as seen by qPCR analysis (Fig. 4). Thus these results suggest that during β AR activation, CBP plays a pivotal role in p53-mediated *PUMA* induction. We further confirmed the relationship between p53 and CBP in the GFP reporter system. Transient transfection of WT MEFs expressing the p53-GFP reporter with a construct encoding wild type CBP consistently gave a shift in fluorescence intensity (in the lower range) whereas similar transfection did not result any shift in fluorescence in *TP53*^{-/-} MEFs (Fig. 5). Moreover, transient transfection of WT MEFs expressing the p53-GFP reporter with a HAT deficient (histone acetyltransferase) mutant CBP [1] did not result in fluorescent shift suggesting that activation of p53 by CBP requires its HAT domain.

Discussion

Increased sympathetic nervous activity in the myocardium is a central feature in patients with heart failure [11]. Aging and chronic stress in humans is associated with marked and sustained increases in the sympathetic nervous system activity in several peripheral tissues such as thymus and heart [10]. This increased activity produces excess catecholamines, which bind β ARs and induce cAMP production. Our previous work points to a role of Bim during cAMP-mediated apoptosis of cells in *in vitro* and in acute, chemically induced cardiomyopathy models [1]. Whilst *BIM*^{-/-} thymocytes and cardiomyocytes were protected against catecholamine-mediated apoptosis [1] in MEFs the protection afforded by deletion of BIM was no greater than in WT cells. Since the protection afforded by deletion of Bax and Bak indicates that the intrinsic apoptosis pathway, regulated by the Bcl-2 protein family is absolutely required for β AR-induced apoptosis, this suggests that additional Bcl-2 family members are involved in this cell death. Our findings using MEFs derived from gene-deleted mice clearly demonstrate that in addition to Bim, Puma contributed to catecholamine-mediated apoptosis, particularly in the earlier phases of apoptosis pathway activation (Fig. 1). It is also clear that as catecholamine exposure persists beyond 24 hours, deletion of Bim and Puma no longer prevents apoptosis, whereas Bax/Bak deletion does. This may indicate that other BH3-only proteins, such as Noxa, become progressively more important over time, or that anti-apoptotic Bcl-2 family member protein levels decline after the first 48 hours.

qPCR analysis showed that *BIM*, *PUMA* and *NOXA* were transcriptionally upregulated in response to catecholamine treatment (Fig. 2). This is similar to the regulation of these pro-apoptotic genes in response to a variety of stress phenomena, including ER stress, growth factor deprivation and gamma-irradiation. In these stress-apoptosis models, p53-dependent transcription plays a key role [30,34,35]. Thus our results that *TP53*^{-/-} MEFs are protected against catecholamine-induced cell death (Fig. 1) supports the notion that the tumor suppressor protein p53 plays an important role in this cell β AR-activated death pathway.

A previous report on β AR-mediated stress response had identified a role for p53 in stress-induced DNA damage [29] and highlighted a mechanism in which β -adrenergic catecholamines, acting through β -arrestin-mediated signalling pathways, suppressed p53 protein levels. Although we could clearly demonstrate β AR-mediated activation of p53- dependent transcription using GFP reporter and through its downstream target p21 levels (Fig. 3A, B), we could not demonstrate any discernable reduction in p53 protein levels (Fig. 3D). This is consistent with the other reports, which suggested a co-operative binding between p53 and the histone acetyltransferase protein CBP [36]. This association is mediated through the interaction between p53 and the KIX domain of p53, which enhances p53 transcriptional activity. Furthermore, direct acetylation of p53 by CBP is known to contribute to its transcriptional activity [37]. This is supported by our data where enforced CBP expression in cells that lack functional CBP was able to drive Puma mRNA expression (Fig. 4). Besides, transient expression of WT CBP, not the HAT-deficient CBP, was able to modulate GFP expression from a *PUMA* promoter in wild type MEFs. These results suggest a direct interlay between CBP and p53 to regulate pro apoptotic protein expression in response to β AR stimulation.

p53 appears to have a differential role in apoptosis induction i.e. in thymocytes and cardiomyocytes it does not play any role and the apoptosis in these cells is mediated solely through CBP-c-Myc-Bim axis [1], and in other cell types such as MEFs (and possibly in other tissues), CBP-p53 axis appears to play a significant role. This discrepancy could be reconciled by the fact that this interaction exists only in selective cell types/tissues. CBP-p53 interaction is a function of p53 phosphorylation status. Multisite phosphorylation represents a mechanism for a graded p53 response, with each successive phosphorylation event resulting in increasingly efficient recruitment of CBP to p53-regulated

transcriptional programs, in the face of competition from cellular transcription factors [38]. Multisite phosphorylation of p53 thus acts as a rheostat to enhance binding to CBP and provides a plausible mechanistic explanation for the gradually increasing p53 response observed following prolonged or severe genotoxic stress or differential role of p53 in cAMP/PKA-mediated apoptotic response in different cell types.

In our previous study on the role of Bim in cAMP/PKA-mediated apoptosis in thymocytes and cardiomyocytes [1], we had suggested that blocking Bim induction could prove to be a therapeutic avenue to treat associated diseases such as cardiomyopathy. However, in light of the present observation, blocking Bim induction alone may not be sufficient. The role of Bim may not be universal as pathologies associated with de-regulated catecholamines such as cancer cachexia [39] could have more to do with p53-mediated apoptosis [40] and thus drug developments should be targeted towards breaking the nexus between CBP and p53.

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Conflict of interest: The authors declare no conflict of interest

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Figure legends

Figure 1. Combined deletion of *PUMA* and *BIM* or *TP53* protects cells to similar degrees from β AR-mediated apoptosis. A. MEFs from different genetic background were treated with 10 μ M Dobutamine and 10 μ M rolipram in serum free medium and apoptosis was measured by annexin V and propidium iodide staining. B. WT or *Puma*^{-/-} MEFs were treated with Dobutamine as in A and protein lysates were subjected to Western blot analysis using anti-Bim or anti-Puma antibodies. Error bars: +/- SEM, n=3. #P<0.005 Vs WT, *BIM*^{-/-} or *PUMA*^{-/-} MEFs and @P<0.05 Vs WT, *BIM*^{-/-} or *PUMA*^{-/-} MEFs.

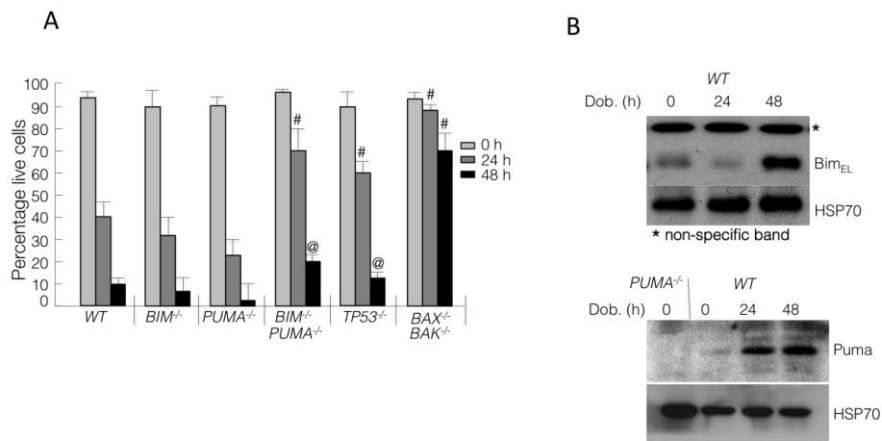
Figure 2. β AR activation leads to transcriptional upregulation of multiple p53 target genes in addition to *BIM*. MEFs were treated with 10 μ M Dobutamine and 10 μ M rolipram in serum free medium and individual transcript levels were measured by qPCR analysis at indicated time points. *NUR77* is used as positive control for cAMP/PKA activation. A. Wild type MEFS and B. *TP53*^{-/-} MEFs. Error bars: +/- SEM, n=3. #P<0.005 Vs untreated control samples.

Figure 3. p53 transcriptional activity but not expression levels is induced during β AR activation. A. Wild type and *T53*^{-/-} MEFs infected with the p53-GFP reporter. Arrows indicate GFP expression levels. B. Dobutamine treatment of the infected MEFs leads to increased p53 activation as shown by the shift in GFP fluorescence. C. Western blot analysis showing increased p21 levels in wild type MEFs during β AR activation and D. Western blot analysis of wild type MEFs showing sustained levels of p53 during β AR activation.

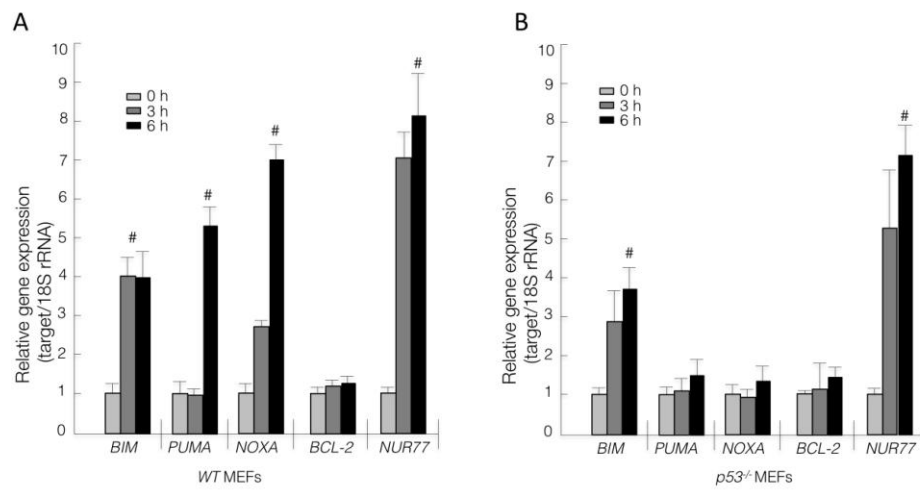
Figure 4. CBP is the mediator of PUMA expression during β AR activation. LK-2 cells were infected with lentiviruses with 4-OHT-inducible expression of CBP (shown in the inset). These cells were treated with dobutamine and rolipram in the presence or in the absence of 4-OHT and transcript levels of PUMA and *NUR77* (positive control) were measured by qPCR analysis. Error bars: +/- SEM, n=3. #P<0.005 Vs untreated control samples.

Figure 5. CBP is a direct activator of p53 transcription. A. Comparative fluorescence analysis of untransfected wild type MEFs (black), wild type MEFs expressing p53-GFP reporter (green) and *TP53*^{-/-} MEFs expressing the p53 GFP reporter (red). B. Comparative fluorescence of wild type MEFs expressing the reporter with and without CBP expression. C. Same as in B, but in *TP53*^{-/-} MEFs and D. Fluorescence analysis in wild type MEFs transiently transfected with a HAT deficient mutant (Y1451C; Ref: 1) of CBP. Figures are representative of two independent experiments conducted in triplicates.

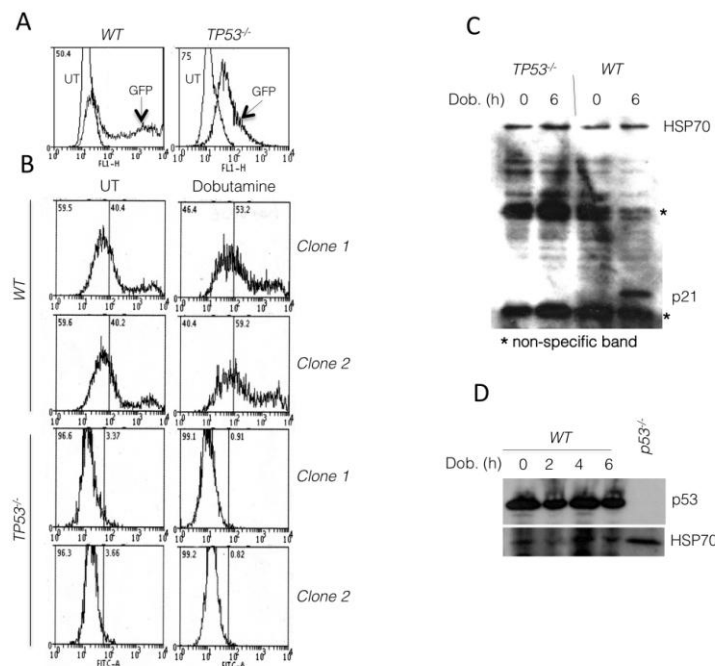
Rahimi et al, Figure 1



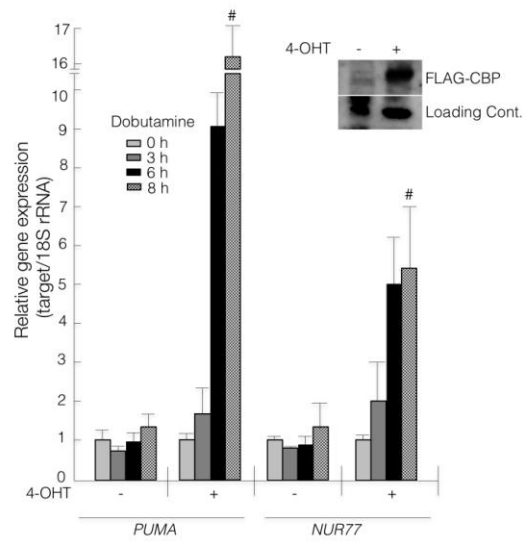
Rahimi et al, Figure 2



Rahimi et al, Figure 3



Rahimi et al, Figure 4



Rahimi et al, Figure 5

