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RESEARCH ARTICLE

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# TAF6 $\delta$ orchestrates an apoptotic transcriptome profile and interacts functionally with p53

Emmanuelle Wilhelm<sup>1</sup>, Mara Kornete<sup>1</sup>, Brice Targat<sup>2</sup>, Jimmy Vigneault-Edwards<sup>2</sup>, Mattia Frontini<sup>3</sup>, Laszlo Torá<sup>4</sup>, Arndt Benecke<sup>2</sup>, Brendan Bell<sup>1\*</sup>

## Abstract

**Background:** TFIID is a multiprotein complex that plays a pivotal role in the regulation of RNA polymerase II (Pol II) transcription owing to its core promoter recognition and co-activator functions. TAF6 is a core TFIID subunit whose splice variants include the major TAF6 $\alpha$  isoform that is ubiquitously expressed, and the inducible TAF6 $\delta$ . In contrast to TAF6 $\alpha$ , TAF6 $\delta$  is a pro-apoptotic isoform with a 10 amino acid deletion in its histone fold domain that abolishes its interaction with TAF9. TAF6 $\delta$  expression can dictate life versus death decisions of human cells.

**Results:** Here we define the impact of endogenous TAF6 $\delta$  expression on the global transcriptome landscape. TAF6 $\delta$  was found to orchestrate a transcription profile that included statistically significant enrichment of genes of apoptotic function. Interestingly, gene expression patterns controlled by TAF6 $\delta$  share similarities with, but are not equivalent to, those reported to change following TAF9 and/or TAF9b depletion. Finally, because TAF6 $\delta$  regulates certain p53 target genes, we tested and demonstrated a physical and functional interaction between TAF6 $\delta$  and p53.

**Conclusion:** Together our data define a TAF6 $\delta$ -driven apoptotic gene expression program and show crosstalk between the p53 and TAF6 $\delta$  pathways.

## Background

Apoptosis is an active program of cell death that is required for normal development and tissue homeostasis in metazoans [1]. The deregulation of apoptotic pathways underlies many human diseases [2]. Consequently, apoptotic pathways represent potential targets for therapeutic control of cell death for diseases including neurodegenerative disorders, autoimmune diseases and cancer [3]. Our previous studies have uncovered the existence of an apoptotic pathway termed the TAF6 $\delta$  pathway that controls cell death [4,5].

TAF6 $\delta$  is an inducible splice variant of the TFIID subunit TAF6 (previously termed hTAF<sub>II</sub>70 or hTAF<sub>II</sub>80). TFIID is a multiprotein complex containing the TATA-binding protein (TBP) and up to 14 evolutionarily conserved TBP-associated factors (TAFs) [6,7]. TFIID is the primary core promoter recognition complex for RNA polymerase II (pol II) and thus plays a key role in the

regulation of transcription of protein-coding genes [8]. The major TAF6 $\alpha$  isoform is ubiquitously expressed [9] whereas strong expression of the TAF6 $\delta$  isoform has only been detected in apoptotic conditions (e.g. HL-60 cells undergoing retinoic acid dependent death) [4]. The use of modified antisense RNA oligonucleotides, also termed splice-switching oligonucleotides (SSO), to experimentally direct the expression of endogenous TAF6 $\delta$  in living cells has recently demonstrated the pro-apoptotic activity of TAF6 $\delta$  [5].

The major TAF6 $\alpha$  isoform contributes to the stability of core TFIID complexes in part by dimerizing with TAF9 via its histone fold domain [9-13]. Structurally, TAF6 $\delta$  differs from TAF6 $\alpha$  only in that it lacks 10 amino acids within its histone fold domain. These amino acids, however, are critical for the interaction of TAF6 $\alpha$  with TAF9 [14], and as a consequence, TAF6 $\delta$  cannot interact with TAF9 [4]. As is the case for TAF9, the highly homologous protein TAF9b cannot interact with the pro-apoptotic TAF6 $\delta$  isoform [15]. TAF6 $\delta$  does retain the capacity to interact directly with other TFIID subunits including TAF1, TAF5, TBP and TAF12.

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Consequently, within cells TAF6 $\delta$  is incorporated into a TFIID-like complex that lacks TAF9 and TAF9b, termed TFIID $\pi$  [4]. Depletion of TAF9 or the highly homologous protein TAF9b in HeLa cells has been shown to alter global gene expression patterns [16]. Presently it is not known whether the transcriptional effects of TAF6 $\delta$  are related to those resulting from the depletion of TAF9 and/or TAF9b. Our previous work revealed that TAF6 $\delta$  can alter gene expression [5], but a physiologically informative definition of the transcriptome impact of TAF6 $\delta$  is currently lacking.

Data documenting a direct interaction between the major TAF6 $\alpha$  isoform with p53 has been shown *in vitro* using recombinant proteins [17], *in vitro* using endogenous human TFIID [18], and in cultured cells using reporter assays [19]. Furthermore, the interaction of TAF6 $\alpha$  with p53 has been shown to be essential for the activation of transcription by p53 *in vitro* [17] as well as *in vivo* in mice bearing point mutations within p53 that block its interaction with TAF6 $\alpha$  [20]. Currently it is not known whether the inducible pro-apoptotic TAF6 $\delta$  isoform can interact with p53. Importantly, TAF6 $\delta$  induces apoptosis in cell lines that lack p53 expression [5]. Moreover, the induction of TAF6 $\delta$  produced similar levels of apoptosis in the HCT-116 p53  $-/-$  colon carcinoma cell line as in its p53 positive counterpart [5]. Thus, TAF6 $\delta$  can induce programmed cell death independently of p53, however the functional relationship between the TAF6 $\delta$  and p53 pathways requires further clarification.

The TAF6 $\delta$  pathway represents a tractable experimental paradigm to elucidate the mechanisms by which human cells respond to their environment through subunit changes in the general transcription machinery [21]. Moreover, there is mounting evidence that the TAF6 $\delta$  pathway may be altered in certain cancers. Aberrant TAF6 expression has been documented in human cancers including lung cancer [22,23] and breast cancer [24,25]. The molecular basis for the induction of apoptosis by TAF6 $\delta$  is currently unknown. In order to shed further light on the impact of TAF6 $\delta$  on the human transcriptome, here we performed a transcriptome-wide analysis of the impact of endogenous TAF6 $\delta$  expression in HeLa cervical carcinoma cells. Our data provide the first physiologically coherent transcriptome signature for TAF6 $\delta$ , establish the relationship of the TAF6 $\delta$  signature with those of TAF9/TAF9b, and identify a functional and physical interaction of TAF6 $\delta$  with p53.

## Results

### The TAF6 $\delta$ orchestrates a pro-apoptotic gene expression program

To establish the impact of TAF6 $\delta$  on global gene expression patterns, the expression of the endogenous

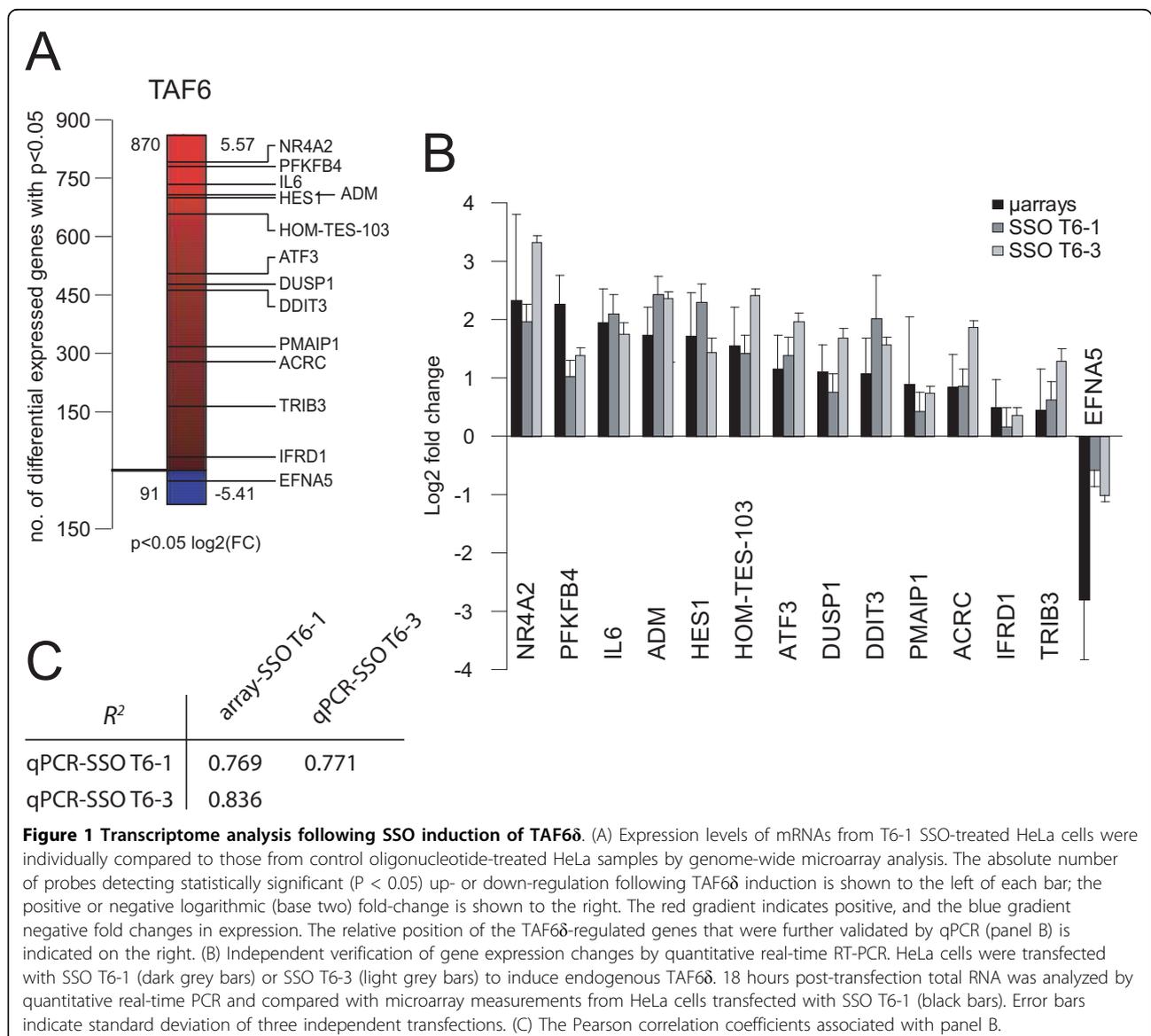
TAF6 $\delta$  splice variant was experimentally induced using splice-switching oligonucleotides (SSO) as previously documented [5]. The HeLa cell line was chosen as a model system for transcriptome studies for three principle reasons. Firstly, these cells are readily transfectable and produce a robust apoptotic response to TAF6 $\delta$ -inducing SSO [5]. Secondly, the TAF6 $\delta$  cDNA was cloned from a HeLa cell library [4] and therefore these cells provide a natural cellular context. Thirdly, HeLa cells express no detectable TAF6 $\delta$  protein under standard culture conditions [5], thus these cells provide a stringently inducible model for SSO studies. To define the impact of TAF6 $\delta$  expression on transcriptome dynamics we took advantage of an experimental approach that combines SSO treatment with high sensitivity microarray analysis [26]. We note that to achieve the statistically significant overrepresentation of gene ontology pathways reported here it was necessary to employ optimized SSO sequences designed to more efficiently induce TAF6 $\delta$  expression than those employed in a previous study [5]. The improved SSO were transfected into HeLa cells and the induction of endogenous TAF6 $\delta$  mRNA and protein was confirmed, as shown in Additional File 1. Total RNA was isolated 18 hours post-transfection and subjected to microarray analysis as previously detailed [26]. Biological triplicates were performed with SSO T6-1 and, as a control to normalize for any non-specific SSO effects, a scrambled oligonucleotide (SSO ctrl). The statistical analysis and filtering of the raw microarray data was carried out as previously described [26] to identify significantly ( $P < 0.05$ ) regulated mRNAs.

The induction of endogenous TAF6 $\delta$  resulted in significant changes in the levels of 961 probes corresponding to 955 independent genes of 27,868 (Figure 1A). Remarkably, 90.5% of the mRNAs significantly changed by TAF6 $\delta$  are upregulated and only 9.5% are downregulated (Figure 1A). These data are consistent with previous results obtained with a less efficient SSO in the HCT-116 cell line [5] and further demonstrate that TAF6 $\delta$  acts primarily as a positive regulator of gene expression. The data also rule out the possibility that TAF6 $\delta$ -induced cell death is a result of a global reduction in mRNA transcription. To validate the TAF6 $\delta$  transcriptome signature we selected 14 genes (and the internal control beta-2-microglobulin gene) for quantitative RT-PCR confirmation. Gene expression changes measured by real-time RT-PCR for the 14 genes showed a strong correlation with changes measured by microarray analysis (Pearson correlation coefficient  $R^2 = 0.769$ , Figure 1B & 1C). To further confirm the specificity of the TAF6 $\delta$  transcriptome signature we employed a distinct TAF6 targeting antisense oligonucleotide (SSO T6-3), whose binding to the

TAF6 pre-mRNA is shifted five nucleotides downstream with respect to SSO T6-1. SSO T6-3 is slightly more efficient in inducing endogenous TAF6 $\delta$  than SSO T6-1 (Additional File 1). Real-time RT-PCR shows that like SSO T6-1, SSO T6-3 also altered the expression of the same 14 TAF6 $\delta$  target genes (Pearson correlation coefficient with microarray measurements  $R^2 = 0.836$ , Figure 1B & 1C). The above data confirm that the microarray results provide an accurate and reproducible measure of the TAF6 $\delta$ -controlled transcriptome landscape.

To examine the specificity of the TAF6 $\delta$ -induced transcriptome signature, we compared the microarray data with those obtained when the pro-apoptotic isoform of a distinct gene, Bcl-x, was induced by SSO under identical conditions [5]. As shown in Additional File 2, the

transcriptome signatures resulting from the induction of TAF6 $\delta$  and Bcl-xS are highly distinct. The vast majority of TAF6 $\delta$ -regulated transcripts (90.5%) were induced while Bcl-xS expression results in a majority of transcripts being repressed (58%). Only a minor fraction (3.4%) of the 870 genes upregulated by TAF6 $\delta$  was also upregulated by Bcl-xS. Of the small number of transcripts repressed (46) by TAF6 $\delta$ , 45 are also repressed by Bcl-xS, possibly reflecting a minor subset of genes that are repressed by both of these pro-apoptotic pathways. The portion of Bcl-xS repressed genes also repressed by TAF6 $\delta$  was minor (15.8%). The fact that genes induced by TAF6 $\delta$  share little overlap with those induced by Bcl-xS underscores the highly specific impact of the TAF6 $\delta$ -inducing SSO on the transcriptome.



To shed light on the mechanisms underlying the pro-apoptotic capacity of TAF6 $\delta$ , we performed gene ontology analysis to identify pathways that are statistically overrepresented within the microarray data (see details in Materials and Methods). Of 131 cellular pathways surveyed, those that are statistically ( $P < 0.05$ ) overrepresented in the TAF6 $\delta$  data set are the Notch, oxidative stress response, integrin, p53, apoptosis and p53 pathway feedback loops 2 pathways (Figure 2A). Genes in the angiogenesis pathway were also overrepresented ( $P = 0.0535$ , Figure 2A) in the TAF6 $\delta$ -induced gene pool. The TAF6 $\delta$ -regulated genes found in the overrepresented pathways are shown in Additional File 3. We next postulated that if the pathways activated by TAF6 $\delta$  represent a physiologically coherent response, then the functional connections between these pathways would not be random. We therefore performed a statistical analysis to identify pathways that share two or more genes activated by TAF6 $\delta$  at frequencies higher than those expected in a random sample. Statistically significant ( $P < 0.05$ ) overrepresentation of shared genes between the integrin and angiogenesis pathways, as well as between the p53 and integrin pathways was observed (Figure 2B). To provide a visual framework that depicts the interconnections between the overrepresented pathways they were mapped onto cellular signaling networks using the Pajek algorithm [27]. This global view reveals that the activated pathways are not randomly distributed and moreover that there is a network of interconnections between TAF6 $\delta$ -regulated pathways, as seen by black points at which distinct colored lines intersect (Figure 2C). Taken together, the results above define a specific TAF6 $\delta$ -driven transcriptome landscape that includes the induction of genes in the Notch, oxidative stress response, integrin, p53, apoptosis, p53 pathway feedback loops 2 and angiogenesis pathways.

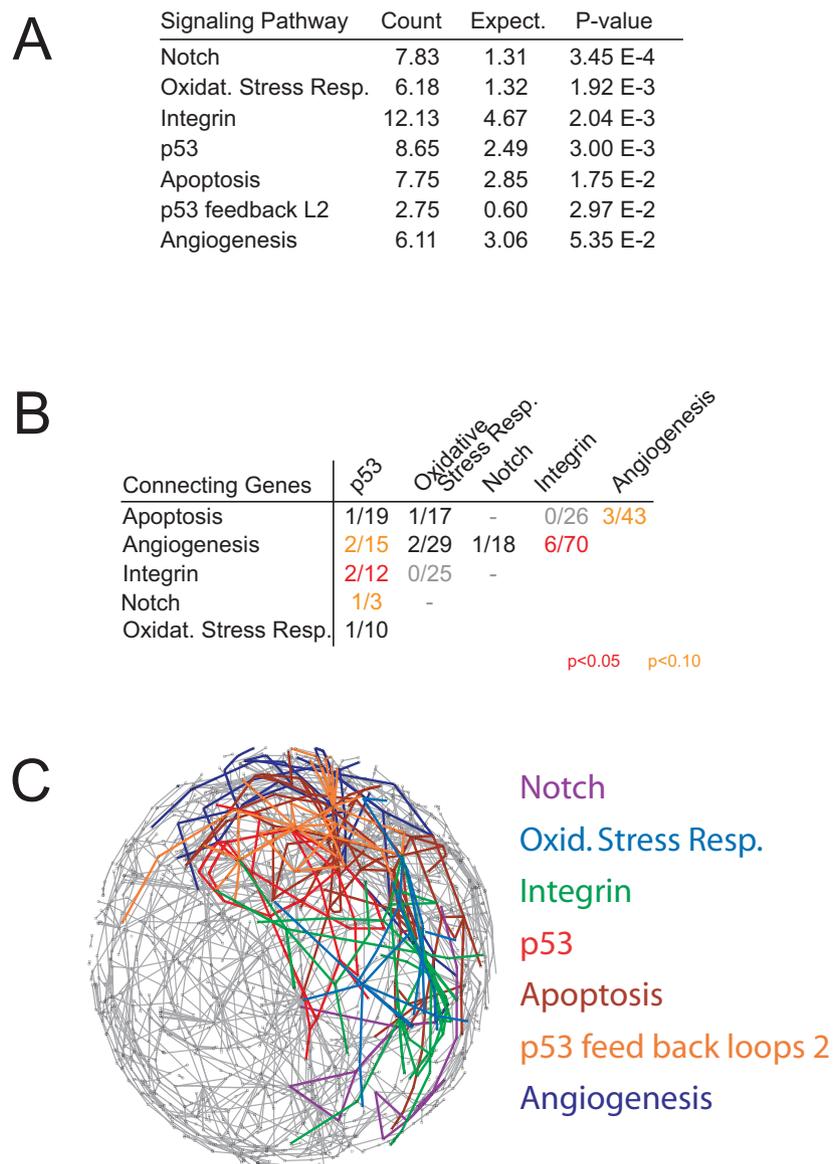
To determine whether or not the changes in mRNA expression in response to TAF6 $\delta$  expression result also in changes in protein levels we selected representative proteins from the TAF6 $\delta$  transcriptome signature for verification by immunoblotting experiments. The microarray data showed expression of ARNT mRNA was repressed by TAF6 $\delta$ , and immunoblotting showed the corresponding ARNT protein levels also decreased in response to TAF6 $\delta$ -inducing SSO (Figure 3A). The levels of the transcription factor TBP were tested as a control for specificity and remained relatively constant in response to TAF6 $\delta$  (Figure 3A). The levels of FOS, JUN, HES1, CDKN2B (p15INK4B), and PMAIP1 (NOXA) were assayed and TAF6 $\delta$  expression resulted in increased protein levels that paralleled increased mRNA levels detected in the microarray experiments in each case (Figure 3A). Importantly, Bcl-x SSO treatment did not result in comparable changes of protein levels,

showing the specificity of their response to TAF6 $\delta$  expression (Figure 3A). These data demonstrate that for the proteins tested the impact of TAF6 $\delta$  on gene expression programs occurs at both the mRNA and protein levels, including the induction of the known pro-apoptotic protein PMAIP1 (NOXA) [28].

Changes in mRNA levels detected by microarray analysis can in principle result from a number of effects including alterations in mRNA stability. To obtain evidence that TAF6 $\delta$  can regulate gene expression in a promoter-dependent and promoter-specific fashion, we tested the ability of endogenous TAF6 $\delta$  to increase target gene expression in luciferase reporter gene assays. We selected four promoters for analysis. The HES1, DUSP1 and ADM promoters were selected since the endogenous *HES1*, *DUSP1* and *ADM* mRNA levels are induced in response to TAF6 $\delta$  expression (Figure 1A & 1B). In the case of HES1, the levels of endogenous HES1 protein were also shown to be induced in response to TAF6 $\delta$  expression (Figure 3A). The 3 selected genes act in several of the pathways activated by TAF6 $\delta$  including the Notch (HES1) [29], angiogenesis (ADM) [30], oxidative stress and p53 pathways (DUSP1) [31-33]. The Bax promoter was also included because it is a p53-responsive and pro-apoptotic gene [34], yet is not induced by TAF6 $\delta$ . The induction of TAF6 $\delta$  in HeLa cells resulted in increased HES1, DUSP1, and ADM promoter-driven gene expression (Figure 3B). In contrast, the Bax promoter was not induced and even measurably repressed (Figure 3B). These results demonstrate that endogenous TAF6 $\delta$  can act directly or indirectly to stimulate transcription in a promoter-dependent manner.

#### **The TAF6 $\delta$ transcriptome signature is not equivalent to those resulting from depletion of TAF9 and/or TAF9b**

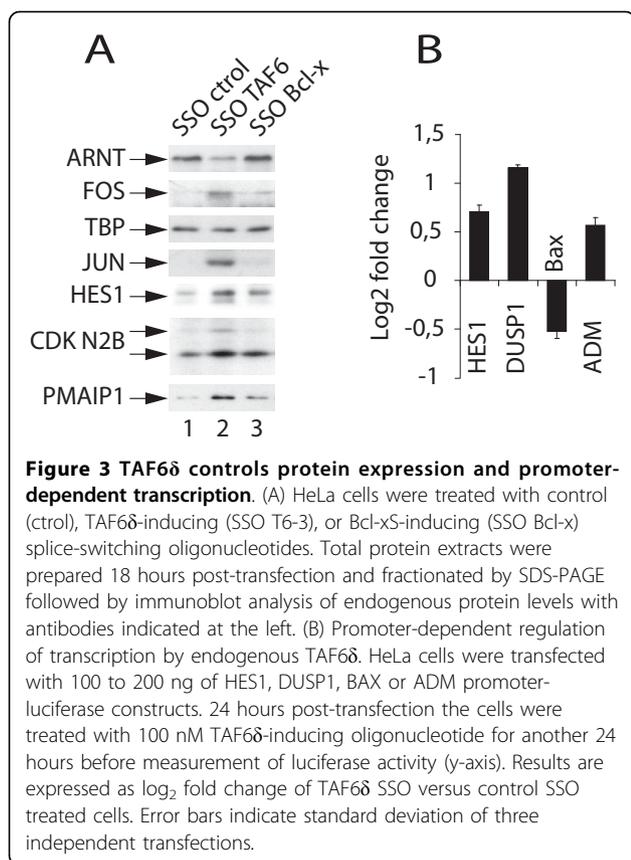
The only currently known functional distinction between pro-apoptotic TAF6 $\delta$  and TAF6 $\alpha$  is that TAF6 $\delta$  cannot interact with TAF9 or TAF9b. We therefore analyzed the extent to which the transcription footprints resulting from TAF6 $\delta$  induction resembles those reported following the depletion of TAF9 and/or TAF9b by treatment with siRNAs in HeLa cells [16]. After mapping of the previous microarray data to enable comparison with our current microarray platform (see Materials and Methods), the datasets were co-filtered to compare transcriptome changes. Of the 961 TAF6 $\delta$ -dependent mRNAs selected for comparative analysis, 803 mRNAs could be mapped between the datasets (Figure 4B). A global view of the magnitude of the changes for these 803 genes showed that changes in response to TAF6 $\delta$  induction were more pronounced than those resulting from TAF9/TAF9b depletion as depicted by heat maps (Figure 4A). 204 mRNAs that are statistically significantly regulated by TAF9 and/or TAF9b were found



**Figure 2 Pathway analysis of the TAF6 $\delta$  transcriptome signature.** (A) Specific cellular pathways are statistically significantly ( $P < 0.05$ ) overrepresented in the TAF6 $\delta$ -regulated transcriptome. "Count" indicates the number of probes within the dataset that corresponds to a given gene ontology pathway, "Expect." is the number of probes one would expect to find for a given pathway in a random dataset of identical size. (B) Interconnectivity analysis reveals statistically significant overrepresentation of shared genes between TAF6 $\delta$ -affected cellular pathways. Red and orange indicate highly significant ( $P < 0.05$ ) and significant ( $P < 0.10$ ) overrepresentation of shared regulated genes, light grey indicates present links of low statistical significance between the pathways from B. (C) Interconnectivity between overrepresented pathways (highlighted in color and listed at right) is shown in a network representation generated using Pajek software [27].

within the TAF6 $\delta$ -regulated transcripts (Figure 4B). Of the 204 genes, 50 showed regulation by both TAF9 and TAF9b. 90 showed regulation by TAF9 alone and 64 showed regulation by TAF9b alone (Figure 4B). To probe for communalities between TAF6 $\delta$ , TAF9 and TAF9b-controlled transcriptomes, pathway analysis was performed on the genes regulated by TAF6 $\delta$  and TAF9 as well as genes regulated by TAF6 $\delta$ , TAF9, and TAF9b. The only pathway that was statistically significantly

overrepresented in these subsets was the "p53 feedback loop 2" ontology (Figure 4C). Pathway analysis was also performed on the TAF9b-dependent gene set alone. Interestingly, the only pathway that was overrepresented was angiogenesis, a pathway also overrepresented in the TAF6 $\delta$  transcriptome signature (Figure 4D). Finally, we compared the effects of TAF6 $\delta$ , TAF9, and TAF9b on single genes from the subset of 50 genes that respond to changes in the expression all of TAF6 $\delta$ , TAF9, and



TAF9b. A majority of genes (~80%) had comparable changes in response to TAF6 $\delta$  induction and TAF9 or TAF9b depletion of which 10 examples are shown in Figure 4D. In contrast, approximately 20% of the TAF6 $\delta$ - TAF9- and TAF9b-dependent genes were positively regulated by TAF6 $\delta$  induction whereas TAF9 or TAF9b depletion resulted in their suppression (Additional File 4). Taken together the analysis shows that TAF6 $\delta$  induction has both overlapping and unique impacts on the transcriptome, when compared to TAF9 and/or TAF9b depletion.

#### TAF6 $\delta$ interacts physically and functionally with p53

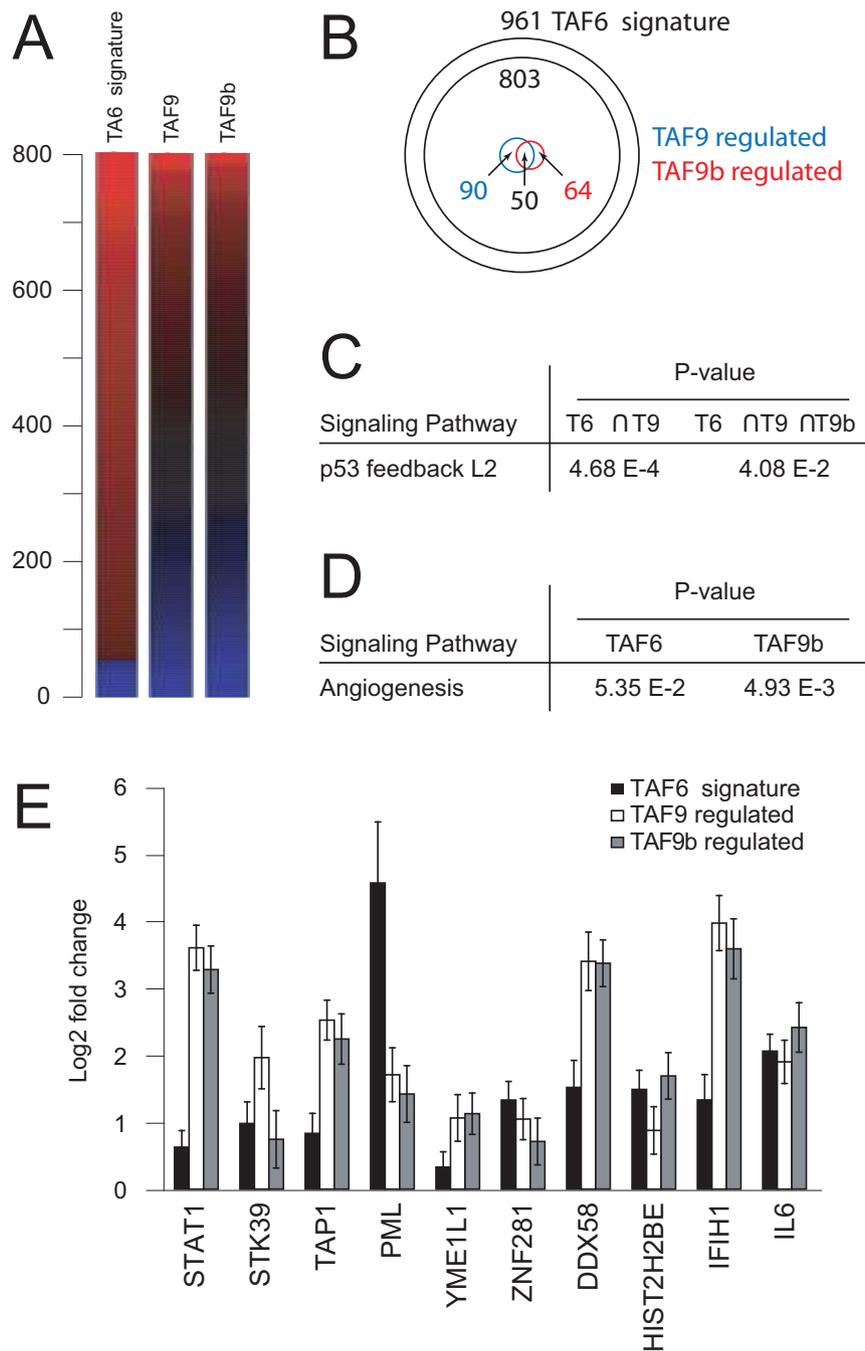
TAF6 $\alpha$  is known to interact with the p53 tumor suppressor protein (see Introduction), but whether the pro-apoptotic TAF6 $\delta$  isoform could retain the capacity to interact with p53 is unknown. Our previous work demonstrated that TAF6 $\delta$  induces apoptosis independently of p53 [5]. Interestingly, the current transcriptome data show that TAF6 $\delta$  induces the expression of several p53 target genes (Figure 2A and Additional File 3). We therefore investigated the capacity of TAF6 $\delta$  to interact with p53. Recombinant Histidine tagged TAF6 $\delta$  was produced in bacteria and purified by nickel affinity chromatography. GST tagged p53 was produced and immobilized on glutathione-sepharose beads. Purified

recombinant TAF6 $\delta$  and TAF6 $\alpha$  were assayed for their capacities to interact with immobilized GST-p53. As previously reported [17], TAF6 $\alpha$  bound efficiently to GST-p53 (Figure 5A, lane 3, upper row). Terminal deoxynucleotidyl transferase (TdT) served as a control for specificity and did not bind to GST-p53 (Figure 5A, lane 3, lower row). Purified His-TAF6 $\delta$  was efficiently retained by GST-p53 (Figure 5A, lane 3, middle row), but not by GST alone (Figure 5A, lane 2). Resistance to high ionic strength buffers can provide a measure of the strength of protein-protein interactions. We therefore challenged the p53-TAF6 interactions with 600 mM KCl washes and found that the interactions were stable in high salt conditions (Figure 5B). These data show a direct and selective interaction between TAF6 $\delta$  and p53 *in vitro*.

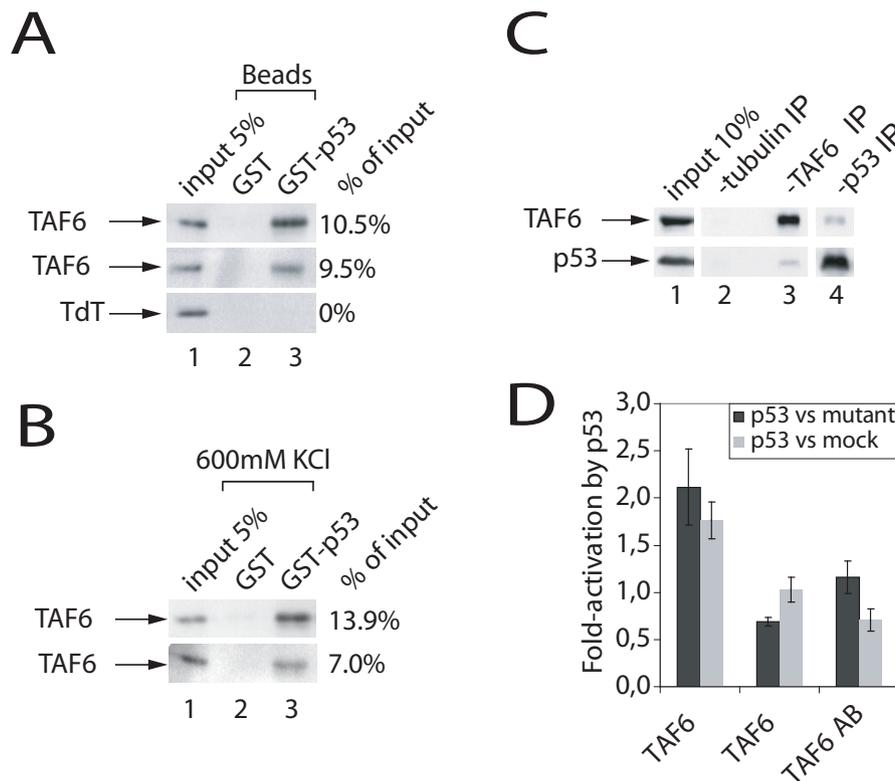
To determine whether the TAF6 $\delta$ -p53 interaction can occur in living cells we performed co-immunoprecipitation assays. As endogenous TAF6 $\delta$  is highly labile and expressed at very low levels [5], TAF6 $\delta$  was expressed by transfection of an expression vector into HCT-116 p53<sup>-/-</sup> cells. Exogenous p53 was provided by co-transfection of an expression vector. Immunoprecipitation of TAF6 $\delta$  resulted in co-immunoprecipitation of p53 (Figure 5C, lane 3), in contrast to the negative control immunoprecipitation of tubulin (Figure 5C, lane 2). In addition, the reciprocal experiment, immunoprecipitation of p53 resulted in recovery of TAF6 $\delta$  (Figure 5C, lane 4). Together, these data show that the interaction between TAF6 $\delta$  and p53 can occur in the cellular context.

We next sought to determine whether the interaction between TAF6 $\delta$  and p53 has functional consequences. We took advantage of the fact that the DUSP1 promoter is activated by TAF6 $\delta$  (Figure 3B) and is also activated by p53 [33]. A reporter construct expressing firefly luciferase under the control of the DUSP1 promoter was co-transfected with p53 expression vectors, as well as vectors expressing TAF6 variants. TAF6 $\delta$  transfection resulted in enhanced DUSP1 expression when co-transfected with p53 (Figure 5D). A truncated version of TAF6 that lacks pro-apoptotic activity [4] failed to show significant co-activation (Figure 5D). The protein levels resulting from transfected plasmids were determined by immunoblotting experiments and are shown in Additional File 5. The data exclude the possibility that higher levels of TAF6 $\delta$  protein (compared to the truncated negative control TAF6) contribute to the activation levels observed. These data show for the first time that TAF6 $\delta$  can interact functionally with p53 to co-activate DUSP1 gene transcription.

Having established that TAF6 $\delta$  can interact functionally with p53, we next sought to define the role of this interaction with endogenous TAF6 $\delta$  and p53. Moreover



**Figure 4 Comparison of the transcriptome effects of TAF6 $\delta$  with TAF9 and TAF9b.** (A) A heat map representation of changes in expression of 800 mRNAs that could be mapped from the published data resulting from depletion of TAF9 or TAF9b [16] to the TAF6 $\delta$ -dependent transcriptome signature shows the global comparison of their respective transcriptome profiles. The red gradient indicates positive, and the blue gradient negative fold changes in expression. (B) A Venn diagram representation of the transcriptome comparison shows the relationship between genes significantly regulated by TAF6 $\delta$ , TAF9 and TAF9b. 803 probes were mapped onto the 961 probe TAF6 $\delta$  signature. (C) Overrepresentation of the "p53 feedback loop 2" gene ontology pathway is common to the TAF6 $\delta$ , TAF9 and TAF9b signatures. Pathway analysis was performed as described in Materials and Methods for genes significantly regulated by both TAF6 $\delta$  and TAF9 (left), by all of TAF6 $\delta$ , TAF9 and TAF9b (right). P-values for overrepresentation are shown in the table. (D) Overrepresentation of the angiogenesis gene ontology pathway is common to the TAF6 $\delta$ , TAF9 and TAF9b transcription signatures. Pathway analysis as in (C). (E) Microarray recorded gene expression changes for examples of genes similarly regulated by TAF6 $\delta$ , TAF9 and TAF9b are shown. Error bars indicate standard deviations.

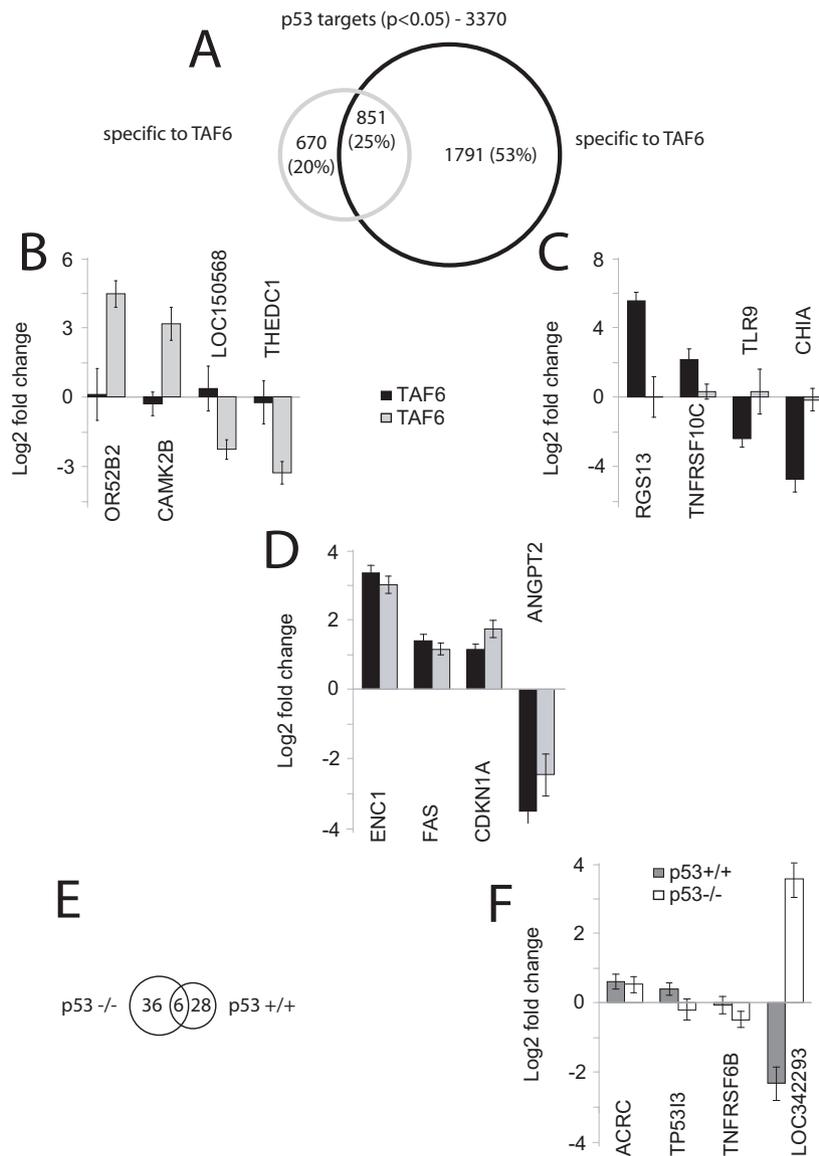


**Figure 5 TAF6 $\delta$  interacts physically and functionally with p53.** (A) TAF6 $\delta$  interacts with p53 *in vitro*. Recombinant purified TAF6 $\alpha$ , TAF6 $\delta$ , or TdT were incubated with immobilized GST-p53. Complexes were washed and retained proteins were analyzed by Western blot. The percentage of retention as quantified by phosphoimager analysis is given at the right. (B) Complexes were washed as in (A) except that the washing buffer contained 600 mM KCl. (C) TAF6 $\delta$  interacts with p53 *in vivo*. HCT116 p53<sup>-/-</sup> cells were transfected with plasmids expressing TAF6 $\delta$  and p53. 28 hours post-transfection protein interactions were assayed by co-immunoprecipitation followed by Western blotting with the appropriate antibodies (Materials and Methods). (D) TAF6 $\delta$  enhances p53-mediated activation of the DUSP1 promoter. HeLa cells were co-transfected with a plasmid expressing the firefly luciferase gene under control of DUSP1 promoter, a plasmid expressing p53 or its mutant R175H or no p53 and various constructs of TAF6. 28 h after transfection, cells were lysed and luciferase activity was measured. Shown are the ratios of relative light unit (RLU) given by cells transfected with p53 relative to cells transfected with p53R175H (black bars) or no p53 (grey bars).

we sought to define the potential crosstalk of TAF6 $\delta$  with p53 upon endogenous genes and at the transcriptome-wide level. We therefore revisited microarray data derived from either wild-type HCT-116 or their p53 negative counterpart HCT-116 p53<sup>-/-</sup> in which endogenous TAF6 $\delta$  expression was induced by SSO (<http://www.ncbi.nlm.nih.gov/geo/> under accession number GSE10795) to test for transcriptional crosstalk between TAF6 $\delta$  and p53. We re-filtered the data specifically to determine the influence of TAF6 $\delta$  versus TAF6 $\alpha$  on p53-dependent genes. The effects of TAF6 isoforms are illustrated in Figure 6A, and reveal three classes of genes. Importantly, 20% of p53-regulated genes (e.g. CAMK2B & THEDC1) change only in the presence of TAF6 $\delta$  (Figure 6B). 53% of the p53-regulated genes change specifically in the presence of TAF6 $\alpha$ , for example TNFRSF10C and CHIA (Figure 6C). 25% of p53-regulated genes, such as FAS and ANGPT2, change expression in the presence of both TAF6 $\delta$  and TAF6 $\alpha$

(Figure 6D). The data show that the expression of TAF6 $\delta$  versus TAF6 $\alpha$  can dictate the outcome of p53-mediated transcriptional signals.

We also filtered the data to determine the reciprocal influence of p53 status upon previously identified TAF6 $\delta$ -dependent mRNAs [5]. 51% of the TAF6 $\delta$ -regulated mRNAs changed significantly only in the absence of p53 (Figure 6E), for example TNFRSF6B (Figure 6F). 9% of the TAF6 $\delta$ -regulated mRNAs changed independently of p53 status, including ACRC (Figure 6F). 40% of TAF6 $\delta$ -regulated mRNAs changed significantly only in cells expressing p53, such as TP53I3 (Figure 6F). In general the influence of p53 on TAF6 $\delta$ -dependent transcription was relatively subtle in magnitude, with at least one exception where the gene LOC342293 displayed opposing regulation in presence or absence of p53 (Figure 6F). Together, the above data establish reciprocal transcriptional crosstalk between the TAF6 $\delta$  and p53 proteins.



**Figure 6 Evidence for transcriptional crosstalk between endogenous TAF6 $\delta$  and p53.** (A) Venn diagram illustrating the partitioning of previously characterized p53-regulated genes [5] into TAF6 $\alpha$  and TAF6 $\delta$  dependent classes. (B) Examples of TAF6 $\delta$  but not TAF6 $\alpha$  dependent p53 target genes. Log base 2 fold changes in gene expression are shown graphically. Error bars show the standard deviations over three independent experiments and gene symbols are shown at the bottom. (C) Examples of TAF6 $\alpha$  but not TAF6 $\delta$  dependent p53 target genes (as in panel B). (D) Examples of TAF6 $\alpha$  and TAF6 $\delta$  dependent p53 target genes (as in panel B). (E) Previously characterized TAF6 $\delta$  target genes [5] partition into p53 dependent and independent genes as illustrated with Venn diagrams. (F) Examples of p53 dependent and independent TAF6 $\delta$  target genes (as in panel B).

## Discussion

The TAF6 $\delta$  pathway has emerged as an apoptotic signaling hub [4,5,21], yet the mechanisms by which TAF6 $\delta$  promotes apoptosis have remained unknown. Here we provide a transcriptome-wide microarray analysis that defines the impact of endogenous TAF6 $\delta$  induction on gene expression patterns. The TAF6 $\delta$  transcriptome footprint showed a predominant role for TAF6 $\delta$  in the activation of gene expression, with

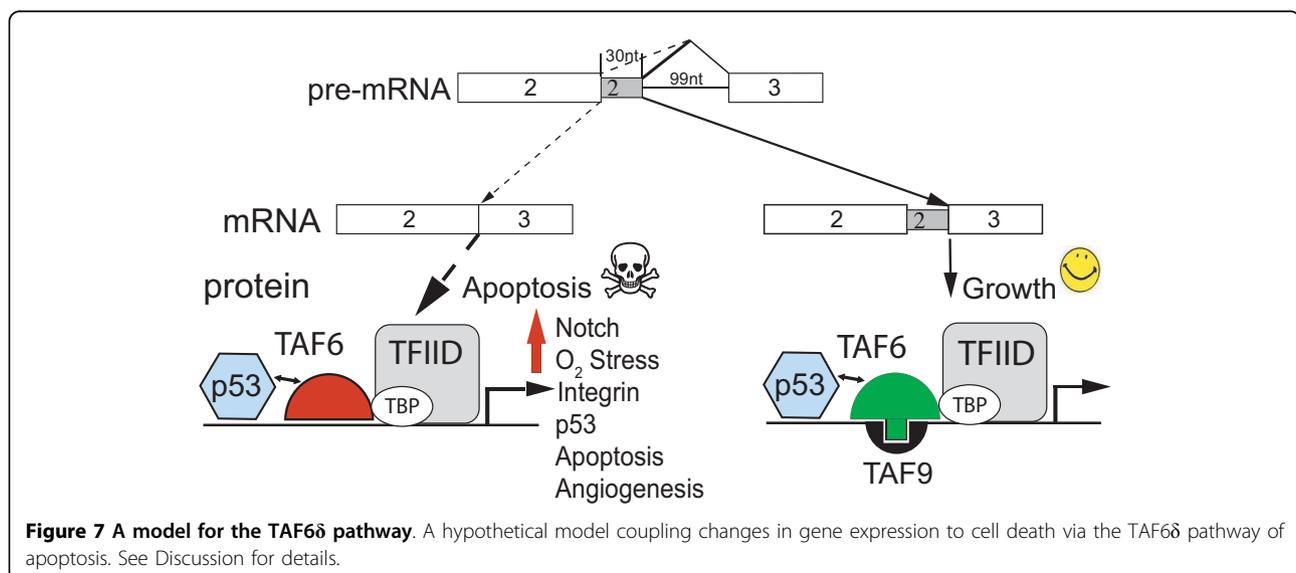
approximately 90% of TAF6 $\delta$ -regulated mRNAs being induced. Genes annotated as belonging to apoptotic pathways were found to be statistically overrepresented in the TAF6 $\delta$ -induced genes. The data therefore provide experimental support for a model wherein TAF6 $\delta$  initiates the apoptotic cascade by inducing pro-apoptotic gene expression. Examples of apoptotic TAF6 $\delta$ -induced genes identified include NOXA and FDXR (Additional File 3) that both code for proteins that each alone

possess pro-apoptotic activity, are p53 target genes, and localize to the mitochondria [35-37]. As TAF6 $\delta$  induces the expression of hundreds of genes, including established pro-apoptotic genes, we propose a model whereby TAF6 $\delta$  represents a signaling hub that transduces apoptotic stimuli to redirect the transcriptional machinery to tip the balance from an anti- to a pro-apoptotic program (Figure 7).

In addition to apoptotic genes the unbiased statistical analysis of our microarray data revealed overrepresentation of genes in the Notch, oxidative stress response, integrin, p53, p53 pathway feedback loops 2, and angiogenesis pathways. The TAF6 $\delta$  pathway is an orphan pathway whose molecular trigger remains unknown. The novel links between these pathways and TAF6 $\delta$  expression provide testable hypotheses for the potential physiological triggers and functions of TAF6 $\delta$ . For example, the identification of p53 target genes prompted us to test and demonstrate a physical interaction between TAF6 $\delta$  and p53 (see below). Within the TAF6 $\delta$ -activated transcriptome signature, unbiased statistical approach showed significant overrepresented interconnections between these individual signaling pathways (e.g. integrin and angiogenesis). Additional support for functional interconnections amongst TAF6 $\delta$ -associated pathways comes from interactions documented in the literature. For example, the Notch [38] and integrin [39] pathways both play important roles in angiogenesis. The fact that several of the TAF6 $\delta$ -induced pathways converge upon the process of angiogenesis implies a physiological coherent impact of TAF6 $\delta$  on gene expression programs. Interestingly, many of the pathways associated with TAF6 $\delta$  expression play roles in the tumor progression. For example,

angiogenesis is both a key event in tumor progression and a target for anti-cancer therapies [40]. Our study therefore provides the rationale to initiate studies to test the impact of TAF6 $\delta$  on the process of angiogenesis *in vivo* in the future.

The incorporation of TAF6 $\delta$  into TAF-containing complexes results in the formation of TFIID $\pi$  that lacks TAF9 (see Introduction). The currently available evidence is consistent with the lack of TAF9 being the only difference between canonical TFIID complexes and TFIID $\pi$  [4], however it is conceivable that the inclusion of TAF6 $\delta$  could cause as yet unknown changes in TFIID $\pi$  subunit composition. TAF6 interacts with TAF9 and the resulting dimeric complex can bind to downstream promoter elements (DPEs) [41,42]. To date our analysis of TAF6 $\delta$ -responsive promoters has revealed no statistically significant enrichment of DPEs or any of the known core promoter element within the promoter regions of genes induced by TAF6 $\delta$  (unpublished data). One mechanistic explanation for the transcriptome impact of TAF6 $\delta$  could be that the loss of TAF9 or TAF9b from TFIID alone drives transcriptional changes. A prediction of this model is that the transcriptome signatures resulting from depletion of TAF9 and/or TAF9b by small interfering RNAs would be highly similar to that resulting from induction of TAF6 $\delta$ . The comparative transcriptomic analysis we provide shows both overlapping and unique features of the TAF6 $\delta$  versus TAF9/TAF9b-dependent transcriptomes. Interestingly, TAF9b-depletion, like TAF6 $\delta$  induction resulted in transcriptome profile with overrepresentation of genes functioning in angiogenesis pathways. Gene ontology analysis of the genes regulated by all of TAF6 $\delta$ , TAF9 and TAF9b showed an overrepresentation of a single ontology



termed the p53 feedback loops 2 pathway, suggesting overlap in the gene expression programs controlled by these TAFs. A limitation of the current study is that the distinct approaches (siRNA versus SSO RNA) and microarray platforms employed results in the loss of information. Nevertheless, clear differences were observed between the transcriptome profiles of TAF6 $\delta$  versus TAF9 and TAF9b (Additional File 4). We conclude that exclusion of TAF9 and/or TAF9b from TFIID results in transcriptome changes that share certain targets with, but that do not fully recapitulate the TAF6 $\delta$  transcriptome signature.

The current transcriptome analysis showed that TAF6 $\delta$  induces genes in the p53 pathway, a result not revealed by previous transcriptome analysis of TAF6 $\delta$  in HCT-116 cells [5]. Based on the finding that TAF6 $\delta$  and p53 can share target genes, we tested and confirmed the direct physical and functional interaction of TAF6 $\delta$  with p53. The impact of endogenous TAF6 $\delta$  on p53-dependent gene expression was further demonstrated at the transcriptome-wide level. The reciprocal capacity of endogenous p53 to influence TAF6 $\delta$ -mediated transcription was also detected, although the magnitude of these effects was globally more modest. Taken together, the data show that there is reciprocal crosstalk between the TAF6 $\delta$  and p53 pathways. The microarray experiments measure changes in expression resulting from both direct and indirect effects of TAF6 $\delta$  and p53. Therefore, the crosstalk we have documented includes that resulting from the direct TAF6 $\delta$ -p53 but also that resulting from indirect transcriptional changes. Given the previous demonstration that TAF6 $\delta$  can induce apoptosis independent of p53 [5], we conclude that TAF6 $\delta$  possesses both p53 independent and p53 dependent activities.

## Conclusion

In summary, we report here that the transcriptome landscape orchestrated by TAF6 $\delta$  includes the induction of apoptotic gene expression. The transcriptome data further uncovered novel links between TAF6 $\delta$  expression and the Notch, oxidative stress response, integrin, p53, p53 feedback loop 2, and angiogenesis pathways. The TAF6 $\delta$ -controlled transcriptome landscape was shown not to be equivalent to those resulting from depletion of TAF9 and/or TAF9b. Finally, the data establish a physical and functional interaction between TAF6 $\delta$  and the p53 tumor suppressor protein.

## Methods

### Cell culture

HeLa cells were grown in DMEM containing 2.5% CS and 2.5% FCS. HCT-116 cells were grown in McCoy's media supplemented with 10% FCS.

### Transfections

2'-O-methyl-oligoribonucleoside phosphorothioate antisense 20-mers were from Sigma-Proligo. "SSO ctrl", "SSO T6-1" [5] and "SSO Bcl-x" [43] have been described. "SSO T6-3" 5'-CUGUGCGAUCUCUUU-GAUGC-3' targets the 3' part of the alternative exon 2 of TAF6. SSOs were transfected at a final concentration of 200 nM with lipofectamine 2000 (Invitrogen) as a delivery agent (1.6  $\mu$ l/ml) according to the manufacturer's recommendations. Plasmids were transfected using 1  $\mu$ l DMRIE-C (Invitrogen) as a delivery agent in a 24 well plate according to the manufacturer's recommendations. All transfections were performed in Opti-MEM medium (Invitrogen).

### Plasmids

Plasmids expressing firefly luciferase under control of the HES1 [29], DUSP1 [33], Bax [44], and ADM [45] promoters have been described. Plasmids expressing TAF6 $\alpha$ , TAF6 $\delta$  and TAF6 $\Delta$ AB [4] and p53 or its mutated form p53R175H [46] have been described. To construct vectors for bacterial production of His-tagged TAF6 $\alpha$  and TAF6 $\delta$  proteins, full length cDNAs were excised from pXJ42-TAF<sub>II</sub>80 $\alpha$  and pXJ42-TAF<sub>II</sub>80 $\delta$ ( $\Delta$ A) [4] respectively, using NotI and XhoI sites. The NotI site was filled in by treatment with Klenow enzyme. The generated fragment was inserted into the SalI and Klenow filled HindIII sites of pQE31 vector (Qiagen), generating pQE31-TAF6 $\alpha$  and pQE31-TAF6 $\delta$  plasmids. pGST-p53Arg [47] and pGEX4-T-3 (GE Healthcare) were used for expression of GST-tagged p53 and GST proteins respectively.

### Antibodies

Monoclonal antibodies directed against TAF6 $\delta$  (37TA-1 & 37TA-2) [4], and TBP (3G3) [48] have been described. The pan-TAF6 monoclonal antibody was purchased from BD Transduction Laboratories. Antibodies against ARNT (sc-17811), JUN (sc-1694), FOS (sc-52), CDKN2B (sc-613) and His probe antibody (sc-803) were purchased from Santa Cruz Biotechnology. Antibodies against HES1 (AB5702), PMAIP1 (Ab13654), alpha-Tubulin (clone B-4-1-2), and p53 (clone PAb1801) were purchased from Millipore, Abcam, Sigma, and Calbiochem respectively.

### RT-PCR

RT-PCR conditions and primers for amplification of both TAF6 $\alpha$  and TAF6 $\delta$  have been described [5].

### Immunocytochemistry

Immunolabelling of TAF6 $\delta$  in fixed cells was performed as described [5].

### Microarray Analysis of Gene Expression

Transcriptome analysis was performed as we previously detailed [26], using the NeONORM normalization method with  $k = 0.20$  [49]. The published microarray data for TAF9 and TAF9b depletion by siRNA were

generated on the Génopole Genomics Platform Strasbourg using custom technology. In order to be able to compare those data directly to data generated from commercial platforms, the unique probe-set identifiers were mapped to non-redundant NCBI and Ensemble gene IDs. Similarly, the ABI700 data generated for this study or from previous studies on an Applied Biosystems Microarray platform were mapped according to the published procedure [50] to the same set of gene IDs. After these mapping procedures >87.9% of unique probe-set or probe IDs could be directly compared which corresponds to > 93.2% comparable genes. For comparative pathway inference analyses the TAF9 and TAF9b data were mapped to, and treated as if ABI700 data to avoid any potential bias stemming from the use of different ontology annotation databases.

Gene Ontology (GO) and KEGG annotations were analyzed using the Panther Protein Classification System <http://www.pantherdb.org> to identify functional annotations that were significantly enriched in the different gene sets when compared to the whole set of genes present on the ABI microarray. Note that a given gene can be assigned to different pathways; in order to reduce multiple probing biases a gene is weighted by the inverse of the number of pathways it can be assigned to, leading to non-natural numbers for the gene counts. P-values are determined using a binominal distribution and a null hypothesis of a random set of genes with identical size. Pathway interconnectivity analysis was performed for the significantly overrepresented pathways based on genes that are annotated to be part of any combination of two of the selected pathways and that were significantly regulated in the subtraction profile analysis. Those numbers were then compared to the entire set of shared genes, and P-values were calculated as above.

Microarray data for the gene sets analyzed herein are provided as Additional Files 3; 6, 7, 8, 9, 10. The transcriptome-wide microarray data for all of the experiments described here were deposited in the M. ACE database <http://mace.ihes.fr> under accession numbers:

TAF9/TAF9b: 2833146766

TAF6 $\delta$  signature: 2937831950; Bcl-x: 2156101006; p53: 2370552334

#### Real time PCR

Real time PCR was performed as described [5]. RNA was prepared with using an RNeasy mini Kit (Qiagen). 1  $\mu$ g of total RNA was reverse transcribed using AMV-RT (Roche). Real-time PCR was performed in a final 25  $\mu$ l reaction on 10 ng of cDNA with 12.5  $\mu$ l 2 $\times$  TaqMan<sup>®</sup> Universal Master Mix (ABI) and 1.25  $\mu$ l of the following 20 $\times$  TaqMan<sup>®</sup> probes: B2M as the internal control (Hs99999907\_m1), ACRC (Hs00369516\_m1), ADM (Hs00181605\_m1), ATF3 (Hs00231069\_m1), DDIT3 (Hs00358796\_g1), DUSP1 (Hs00610256\_g1), EFNA5

(Hs00157342\_m1), HES1 (Hs00172878\_m1), HOM-  
TES-103 (Hs00209961\_m1), IFRD1 (Hs00155477\_m1),  
IL6 (Hs00174131\_m1), NR4A2 (Hs00428691\_m1),  
PFKFB4 (Hs00190096\_m1), PMAIP1 (Hs00560402\_m1),  
or TRIB3 (Hs00221754\_m1).

#### Luciferase assays

Cells were washed with PBS and lysed with Passive lysis buffer (Promega). The luciferase activity was measured on a Lumistar luminometer (BMG Labtech), after injection of 2 $\times$  Luciferin reagent; 270  $\mu$ M CoenzymeA, 470  $\mu$ M D-Luciferin, 530  $\mu$ M ATP (all from Sigma-Aldrich) 40 mM Tris-Phosphate pH 7.8, 2.14 mM MgCl<sub>2</sub>, 5.4 mM MgSO<sub>4</sub>, 0.2 mM EDTA, 33.3 mM DTT.

#### Recombinant protein production

Escherichia coli M15 cells were transformed with plasmids expressing His-TAF6 $\alpha$  and His-TAF6 $\delta$  fusion proteins and grown to log-phase before induction of protein expression with 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) for 18 h at 16.5°C. The cell pellets were resuspended in Ni buffer composed of 1 M NaCl, 30 mM Tris pH8.0 and supplemented with 25% glycerol and 1 $\times$  Complete protease inhibitor cocktail (PIC) (Roche) and 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and sonicated twice for 5 minutes on ice. After clarification, the supernatant was loaded on a His-trap column (GE Healthcare) equilibrated in Ni buffer containing 10 mM imidazole. The column was sequentially washed with Ni buffer containing 60 mM and 100 mM of imidazole and the proteins were finally eluted with 250 mM imidazole. Purified proteins were dialysed against buffer D (20 mM HEPES pH7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA).

GST and GST-p53 fusion proteins were produced in Escherichia coli BL-21 cells by IPTG induction (1 mM) of a log-phase culture for 3 h. The cell pellets were resuspended in PBS containing 0.5% Triton X-100 and sonicated twice for 2 minutes. After clarification, the supernatant was incubated with glutathione-sepharose (GE Healthcare) for 1 h 30 at 4°C. The beads were washed three times in PBS and once in buffer D supplemented with 5 mM MgCl<sub>2</sub>, 0.1% NP40 and EDTA up to 1 mM (buffer D+).

#### Protein-protein Interactions

GST "pull-down" assays were performed essentially as previously described [17]. GST- and His-tagged proteins were pretreated with 0.5 U DNase (Promega) for 10 minutes at 37°C before the interaction assay. Equal amounts of GST or GST-p53 linked to sepharose beads were then incubated with His-TAF6 $\alpha$ , His-TAF6 $\delta$  or His-TdT for 1 hour at room temperature in buffer D supplemented with 0.5  $\mu$ g RNase A (USB) (buffer D+). After four washes with buffer D+ containing 100 mM or 600 mM KCl, bound proteins were analyzed by SDS-PAGE and immunoblotting.

## Immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris pH8, 1% NP40, 0.25% Na deoxycholate, 150 mM KCl, 1 mM EDTA) supplemented with 1× PIC and 0.5 mM PMSE. The lysate was diluted 1/10 with IP100 buffer (25 mM Tris pH8, 5 mM MgCl<sub>2</sub>, 10% glycerol, 100 mM KCl, 0.1% NP40, 0.3 mM DTT, PIC, PMSE) and precleared with protein G-sepharose beads for 2 hours at 4°C. The precleared lysate was then incubated overnight at 4°C with anti-p53, anti-TAF6δ or anti-tubulin antibodies immobilized on protein G-sepharose beads. After extensive washes with IP100 buffer, complexes were analyzed by SDS-PAGE and immunoblotting.

### Additional file 1: Splice-switching oligonucleotide (SSO) targeting of TAF6.

(A) The region of the TAF6 pre-mRNA that includes two alternative 5' splice sites (SSs) that produce either the constitutive α splice variant or the alternative δ splice variant is schematically depicted. Selection of an intron-proximal α 5' splice site (SS) results in production of the α isoform of TAF6 (at right) whereas the selection of the proximal δ 5' SS results in the production of the δ isoform (at left). The SSO oligonucleotides (SSO T6-1 & SSO T6-3) base pair with the alternative exon to force splicing from the distal 5' SS and enforce expression of the endogenous TAF6δ isoform (at left). The protein produced by the major splice variant, TAF6α, can interact with the TFIID subunit, TAF9 via its histone fold domain. In contrast, TAF6δ lacks 10 amino acids of helix 2 of its histone fold motif and therefore cannot interact with TAF9. (B) SSO T6-3 induces endogenous TAF6δ mRNA expression. HeLa cells were transfected with antisense oligonucleotides: SSO Ctrl, SSO T6-1 or SSO T6-3. 24 hours post-transfection total RNA was isolated and subjected to RT-PCR with primers that amplify both the TAF6α and the alternative TAF6δ mRNAs. (C) Quantification of TAF6δ expression. Black bars show the percentage of TAF6δ over total TAF6 mRNA as amplified by RT-PCR as in B and separated by microfluidity and analyzed using a 2100 Agilent Bioanalyzer. Light grey bars show the percentage of TAF6δ expressing cells after SSO transfection as in B except that cells were fixed and stained with an anti-TAF6δ antibody for immunocytochemistry (ICC) and a minimum of 500 cells were scored for their staining with an anti-TAF6δ antibody.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S1.EPS>]

### Additional file 2: The transcriptome impact of TAF6δ-inducing splice switching oligonucleotides (SSO) is highly distinct from the impact of Bcl-x SSO.

TAF6δ-inducing SSO microarray data have been compared with previously documented Bcl-x SSO data [26]. Venn diagrams show the number of distinct and overlapping genes in the up-regulated (top) or down-regulated (bottom) gene subsets resulting from treatment with TAF6δ versus Bcl-x SSO.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S2.EPS>]

### Additional file 3: TAF6δ Signature.csv.

This comma separated value data file contains a tabular listing of the TAF6δ target genes identified in this study and their annotation.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S3.CSV>]

### Additional file 4: Differential regulation of gene expression by TAF6δ versus TAF9/TAF9b.

Log base 2 fold changes in gene expression are represented by black (TAF6δ-regulated), white bars (TAF9-regulated), or grey (TAF9b-regulated) bars. Error bars show the standard deviations over three independent experiments and gene symbols are shown at the bottom.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S4.PS>]

### Additional file 5: Proteins levels resulting from transfection of TAF6 and p53.

HeLa cells were transfected with plasmids expressing TAF6, p53 or p53 bearing the R175H mutation, and DUSP1-luciferase reporter constructs. (A) Total protein extracts were prepared 28 hours post-transfection and fractionated by SDS-PAGE followed by immunoblot analysis of exogenous protein levels with antibodies indicated at the left. A representative immunoblot is shown. (B) Protein levels from three independent transfections were quantitated by phosphoimager analysis and normalized to Actin levels. Error bars show the standard deviations.

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### Additional file 6: TAF9 Signature.csv.

This comma separated value data file contains a tabular listing of the TAF9 target genes identified in this study and their annotation.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S6.CSV>]

### Additional file 7: TAF9b Signature.csv.

This comma separated value data file contains a tabular listing of the TAF9b target genes identified in this study and their annotation.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S7.CSV>]

### Additional file 8: TAF6δ TAF9 common genes.csv.

This comma separated value data file contains a tabular listing of the common TAF6δ and TAF9 target genes identified in this study and their annotation.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S8.CSV>]

### Additional file 9: TAF6δ TAF9b common genes.csv.

This comma separated value data file contains a tabular listing of the common TAF6δ and TAF9b target genes identified in this study and their annotation.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S9.CSV>]

### Additional file 10: TAF6δ TAF9 TAF9b common genes.csv.

This comma separated value data file contains a tabular listing of the common TAF6δ and TAF9 and TAF9b target genes identified in this study and their annotation.

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[<http://www.biomedcentral.com/content/supplementary/1471-2199-11-10-S10.CSV>]

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#### Authors' contributions

EW performed or directed all of the experiments with the exception of microarray analysis. MK performed the reporter gene experiments. BT and JVE performed and analysed the microarray data. MF and LT generated and provided microarray data on TAF9/TAF9b. BB and AB conceived and designed the experiments and wrote the manuscript. All the authors read and approved the final manuscript.

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