FEMS Yeast Research Advance Access published September 27, 2016

Human transcription factors in yeast: the fruitful examples of P53 and NF-κB.

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Abstract

The observation that human transcription factors (TFs) can function when expressed in yeast cells, has stimulated the development of various functional assays to investigate i) the role of binding site sequences (herein referred to as Response Elements, REs) in transactivation specificity, ii) the impact of polymorphic nucleotide variants on transactivation potential, iii) the functional consequences of mutations in TFs, and iv) the impact of cofactors or small molecules. These approaches have found applications in basic as well as applied research, including the identification and the characterization of mutant TF alleles from clinical samples. The ease of genome editing of yeast cells and the availability of regulated systems for ectopic protein expression enabled the development of quantitative reporter systems, integrated at a chosen chromosomal locus in isogenic yeast strains that differ only at the level of a specific RE targeted by a TF or for the expression of distinct TF alleles. In many cases, these assays were proven predictive of results in higher eukaryotes. The potential to work in small volume formats and the availability of yeast strains with modified chemical uptake have enhanced the scalability of these approaches. Next to well-established one-, two-, three- hybrid assays, the functional assays with non-chimeric human TFs enrich the palette of opportunities for functional characterization. We review about twenty-five years of research on human sequence-specific TFs expressed in yeast,

with an emphasis on the P53 and NF-κB family of proteins, highlighting outcomes, advantages, challenges, and limitations of these heterologous assays.

Using yeast to study human sequence-specific TFs. Transcription is a process involving dynamic, spatial and temporal organization of TFs and cofactors that modulate the recruitment of RNA Polymerases on chromatin regions to initiate transcription or to regulate transcription rates as part of the responses to specific stimuli [127]. Most of the involved *trans-acting* factors recognize specific *cis-acting* elements in the form of DNA codes, called REs, consisting of ~6-10 nucleotide motifs with various degrees of variability at individual positions [94], usually summarized by Position Weight Matrices (PWM) or logos [19, 111]. Many studies have drawn a correlation between the identity of nucleotides at specific positions within an RE and relative binding behaviour of a TF. Various tools are available to predict the closeness of input DNA sequences to TFs consensus REs, such as Jaspar (http://jaspar.genereg.net/) and Tomtom (http://meme-suite.org/tools/tomtom). However, DNA binding affinity may not accurately predict the transcriptional responsiveness of a promoter, especially for TFs that are active as dimers or tetramers. High-throughput assays, including ChIP-sequencing, ChIP-exonuclease and approaches for the parallel measurement of enhancer reporters, are identifying TF REs and establishing complex rules for the correlation between TF occupancy at chromatin sites and transcription rates of proximal or distant genes [71, 87, 101, 114, 140].

The yeast *S. cerevisiae* is a recognized model system for understanding aspects of the biology of human proteins using complementation assays, ectopic expression and functional assays even for the cases where a clear orthologous yeast gene is absent [38, 53, 109, 132]. As summarized here, many human TFs can act as such when ectopically expressed in yeast cells where, thanks to the evolutionary conservation of basic components of the transcription machinery [57], they can modulate the expression rates of a reporter gene by acting through promoters engineered to contain appropriate REs. In fact, basal factors (e.g. TATA-Box binding proteins and the largest subunit of RNA Polymerase II) and chromatin modifying complexes (e.g. Histone Deacetylases) are well conserved between yeast and human cells [57]. However, chimeric constructs, where the full-length cDNA of human TFs (*e.g.* NKX2-5 or HAND1 [41,

102]) was fused to a transactivation domain of the acidic class derived from P53, showed higher transactivation potential, with no appreciable changes in transactivation specificity [67]. This result is in line with the observation that not all types of mammalian transactivation domain interact proficiently with the yeast transcription machinery [57].

Inducible, repressible or constitutive promoters allow to express human TFs in yeast [145] [5] [86] (Supplementary Table 1). In particular, the use of the inducible *GAL1* promoter (that can be finely tuned in its transcription rates by changing the amount of galactose in the media) represented a critical development for studying P53 and NF-κB family proteins [43]; at the same time basal expression of the TF from the *GAL1* promoter can be achieved using raffinose as a carbon source. Furthermore, the bidirectional *GAL1,10* promoter can also be exploited to co-express two TFs from the same construct [113].

Different reporter systems have been developed in the yeast *S. cerevisiae* to evaluate transcriptional responses (especially P53 family-dependent) including qualitative and quantitative assays (Supplementary Table 2). Qualitative or semi-quantitative reporter systems were based on the *URA3, HIS3* or *ADE2* reporter genes [11, 27, 61, 116, 133]. The TF-dependent expression of these reporter genes confers uracil, histidine and adenine prototrophy. Furthermore, the *URA3* reporter allows a counter-selection, *i.e.* the selection for cells not expressing functional URA3: in fact, in the presence of 5-fluoro-orotic acid (5FOA), cells expressing functional *URA3* will convert this molecule into a toxic compound and will not survive**.** The *ADE2* reporter system has the advantage that, besides the nutritional selection, a colorimetric phenotype can be used to discriminate yeast cells containing a wild-type (*i.e.* functional) or a mutant (*i.e.* not functional) TF. In fact, on plates containing low concentrations of adenine (2.5-5 mg/L) cells that transcribe the *ADE2* gene grow as normal size, white colonies while those not expressing the reporter gene (or expressing it at low level) generate small red (or pink) colonies due to the accumulation of a coloured intermediate in the biosynthetic pathway of adenine. The *ADE2* reporter has been also exploited to select for REs resulting in white colonies on plates containing low adenine amount or even allowing for growth on plates lacking adenine entirely. This context allowed the selection of high activity P53 REs, at low P53 protein levels where a typical P53 RE would not result in transactivation [40, 52]. The semi-quantitative color-based *ADE2* reporter gene has been replaced with the quantitative Firefly Photinus pyralis [43] or the Renilla reniformis luciferase genes (LUC) [113]. The production of fluorescent proteins

such as EGFP (Enhanced Green Fluorescent Protein) or DsRed (Discosoma sp. red fluorescent protein) has been also used for quantitatively monitoring the transcriptional regulation by all possible amino acid substitutions (caused by a point mutation) throughout P53 protein [55].

A particularly versatile approach in the yeast *S. cerevisiae* is *Delitto Perfetto* that consists in an oligonucleotide targeting approach employed to rapidly target desired REs upstream of a minimal promoter to control the expression of the reporter gene [43, 136]. The experiment takes 5-6 days to complete and the targeting of the chosen locus is achieved using single strand oligonucleotides or *in vitro* extended partially complementary oligonucleotides, or also PCR products. The linear molecules must contain at least 30 nucleotides of homology on both ends for the chosen chromosomal region [129]. The homology sequences must correspond to sequences flanking the site of integration of a double marker cassette (ICORE), containing the genes *KlURA3* (counter-selectable by 5FOA) and *kanMX4* (reporter providing resistance to Geneticin, G418), previously integrated at the same locus. Such integration is achieved by a PCR approach, using primers that contain tails of homology (50 nucleotides long) for the chosen region. The ICORE cassette can be amplified from the plasmids developed by Dr. Storici (see reference [130], for details of different versions of the cassette). The cassette also contains the cDNA for the homing endonuclease I-SceI under an inducible promoter (*GAL1,10*) and the unique target site for this enzyme. Hence, prior to transformation of yeast with the targeting sequence, I-SceI expression is induced by galactose resulting in a single double strand break at the site of ICORE integration that greatly enhances the frequency of targeting events, which are selected on plates containing 5FOA (1g/L). Colonies growing on 5FOA plates are replica plated both on rich YPDA and on YPDA containing G418 (0.4mg/L) to identify those that are also sensitive to the antibiotic, hence candidates of a targeting event. The integration of the desired RE is confirmed by colony PCR and Sanger sequencing. The process is highly efficient, with more than 1000 replacement events obtained with a single transformation. Through this technique, a plethora of isogenic yeast strains differing only in at least one nucleotide in a RE placed in the promoter region of the appropriate reporter gene were constructed and utilized. The combination of tuneable promoters for TF allele expression and of the isogenic reporter strains led to the development of a matrix of results where the only variables are the amount of TF protein and the nature of its cognate target RE in a completely isogenic system [99]. By focusing on wild-type TFs families, the results delineate the molecular rules (e.g. DNA code) that determine their

ability to transactivate the reporter gene. A parallel approach with mutant TFs allows the characterization whether a mutant TF has lost completely its transactivation ability or whether it can still have residual activity on specific REs, a result which can be pharmacologically relevant.

Moreover, while the basic set-up of the yeast experimental system focuses on aspects of *cis*-regulation, including the impact of TF mutations and RE sequence variants, attempts have been made to adapt yeast-based transcription assay to evaluate the impact of protein cofactors and for testing small molecules targeting human TFs (Figure 1) [4, 113].

Determining how nucleotide sequences variations of REs govern the transactivation potential of P53 or NF-κB TFs family

A. P53

The mammalian P53 is a tetrameric (dimer of dimers) TF. Each P53 dimer recognizes an RE of 10 nucleotides (RRRCWWGYYY, R=A or G; W=A or T; Y=C or T) [23]. Two such half-site motifs comprise a full site P53 RE. The half-sites can be either adjacent or spaced apart by up to 13 or even ~20 nucleotides without large changes in DNA binding affinity measured *in vitro* with naked DNA [76, 78]. However, results from functional assays in various systems, competitive DNA binding assays in solution, and P53 occupancy studies in chromatin consistently showed higher affinity and transactivation for REs that do not have spacer between the two decameric motifs [15, 123]. In terms of location in the genome, P53 REs are generally embedded in the promoter or proximal enhancer regions of established P53 target genes [23], but there are many examples of REs in intronic regions, distal enhancers, super-enhancers [108], and in repetitive elements [149]. It has been proposed that besides mediating transcriptional regulation, P53 REs may serve as chromatin accessibility factors contributing to P53-mediated genome stability [106]. Since the full consensus P53 RE motif is very degenerate $(RRRCWWGYYY)_2$, it follows that individual REs can differ in sequence by one to several nucleotides, potentially impacting on DNA binding affinity. In addition, well-established P53 REs often contain up to three non-consensus bases -herein defined as mismatches- that can further contribute to differences in DNA binding affinity. Hundreds of P53 target genes have been identified in the human genome [76, 78], and indeed virtually all P53 REs are non-identical in sequence; moreover, it appears there has been significant evolutionary divergence among P53

REs from different species [36, 49]. Hence, it has been hypothesized that the specific nature of the P53 RE can directly impact on transactivation specificity to the point that specific types of REs would have been selected for, in the promoters of P53 target genes involved in different biological responses, for example cell cycle arrest or apoptosis [2, 143].

The analysis in yeast of many variants of the P53 RE at a constant genomic location enabled the deconstruction of the P53 RE and established the impact of nucleotide variants, spacer and organization of RE half sites on transactivation capacity (Figure 2, A1) (Table 1). Recently, all the information on sequence features of P53 REs and resulting transactivation potentials measured in yeast were coded in a pattern search algorithm (p53 Retriever) that maps, and then ranks, canonical P53 full sites, as well as non-canonical REs, according to their predicted transactivation potentials [135].

Taken together, all the studies on P53 REs establish P53 as a highly pleiotropic "master regulator" in virtue of its capacity to alter the expression of a plethora of downstream genes, potentially leading to the activation of different response pathways and cellular outcomes, and the RE sequence as a first regulatory layer in achieving transactivation specificity.

Definitely, the approach in yeast has limitations in modelling the accessibility of REs and their interactions with other nearby *cis-elements*, which can be also important factors in establishing P53-dependent transactivation at target genomic sites. For example, P53 half-sites can synergize with other binding sites such as Estrogen Receptor (ER) half-sites in human cells, while in yeast only additive effects were observed [14, 77].

The concept of mutant P53s functional heterogeneity emerged in its full-blown complexity from extensive studies performed in yeast

In human cancers *TP53* gene is mainly altered by single amino-acid substitutions affecting preferentially six major hotspot residues within the DNA-binding domain (DBD) of the P53 protein (R175, G245, R248, R249, R273 and R282), even though more than 1800 different amino-acid changes have been reported, some of which have been observed infrequently in tumors [65]. Mutant P53s have been classified as DNA contact or structural mutants, based on the effect of the amino-acid substitution on the interaction with DNA (e.g. p.R273H) or on the protein structure itself (e.g. p.R175H) [13, 58, 59]. From the functional point of view, single

amino-acid substitutions appeared to differentially affect P53 functions, having the potential to generate a wide range of phenotypic diversity, which could impact on important clinical features, such as tumor aggressiveness, chemo-resistance and metastatic potential [85, 93, 141].

The concept of mutant P53s heterogeneity (Table 2) emerged in its full-blown complexity through a great amount of experimental data on the functional impact of *TP53* missense mutations, generated in the last 15 years, and now freely available in mutation databases (p53.free.fr/; p53.iarc.fr/) [65]. Specifically, the TP53MUTLOAD (MUTant Loss Of Activity Database) dataset is dedicated to a detailed analysis of the properties of each mutant P53, comprising transactivation, cell growth properties, change of conformation, localization and gain of functions properties. Moreover, functional assays have been performed by many laboratories, in yeast as well as in human cells using reporters to measure different properties of mutant P53s including: i) transactivation potential; ii) temperature sensitivity; iii) dominant negative effect over the wild-type protein; iv) functional modulation of other members of the P53 family (P73 and P63), usually reported as interference, and v) interactions with other TFs [11, 14, 55, 77, 79, 80, 104, 117] (Figure 2, B1).

The original assays exploited a growth marker, such as *HIS3* or the growth and colour, in case of the *ADE2* gene, whose transcription is placed under wild-type P53 control by an engineered promoter [12, 45, 109, 120-122]. For example, lack of *ADE2* expression resulting from the expression of loss-of-function human P53 cDNA allele would lead to small red yeast colonies on plates containing low adenine amounts. Conversely, P53-dependent *ADE2* expression results in yeast cells becoming proficient for adenine biosynthesis and giving rise to large white colonies. This rapid colour assay is combined with a gap repair approach so that each transformant colony growing on the selective media has captured and constitutively expresses a single P53 cDNA allele, obtained from RT-PCR of human RNA samples, including cancer biopsies, hence the FASAY acronym (Functional Analysis of Separated Alleles in Yeast). The basic protocol of FASAY has been refined over the years. For example, in case of low quality RNA, the PCR amplification of the P53 cDNA is difficult, causing a dramatic drop in the quality of the results. To overcome this inconvenient, a split version of the gap repair assay was introduced. The entire P53 cDNA is amplified as two overlapping fragment that are evaluated separately. Since shorter cDNA amplicons are needed, this upgraded version of the assay is more efficient with low quality RNA [146]. A second improvement consisted in the introduction of

different P53-responsive promoters using different versions of P53 REs controlling the transactivation of the reporter gene, which led to the identification of P53 mutants able to discriminate among different REs [43, 104].

Transactivation has been the most extensively studied biochemical function of mutant P53s. Work of several groups, including our own, established that nearly all mutant P53s at hotspot residues have lost or retain only a very weak transactivation function (Loss of Function, LoF, or Severe Deficiency, SD mutant P53s [82, 83]). Nevertheless, a significant fraction of tumor-associated mutant P53s, that hit other positions of the P53 protein and are generally found at moderate to low frequency in cancer [126] retains some level of transactivation potential and can discriminate between the P53 REs in yeast reporter assays (Partial Function or Partial Deficiency, PD mutant P53s) [11, 51, 104], among P53 target genes in cell lines [69] and also *in vivo*, in mouse models [68].

The most comprehensive functional study examined 2.314 mutants, virtually all missense mutations that result from single nucleotide substitutions in the P53 coding sequence. The transactivation activity of mutant P53s was characterized towards eight different P53 REs using yeast reporter strains [55].

From the functional point of view, other categories of mutant P53s, such as "altered specificity", "super-trans" or "intragenic-suppressor", that are virtually absent from the P53 mutation spectrum in cancer, have been identified in yeast. These mutations proved to be useful tools to study mechanisms underlying transactivation specificity [9, 40, 42, 104]. Interestingly, the majority of these mutant P53s, that can also exhibit enhanced DNA binding affinity and transactivation activity with respect to wild-type P53, lie in the L1 loop, a highly conserved portion of the P53 DBD that is a "cold spot" for mutation in cancer [75, 107].

The ability to study the transactivation properties of P53 protein in yeast has been exploited also for clinical research. It is known that germline mutations at the *TP53* gene are responsible for cancer proneness syndromes ranging from the more severe known as Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL), to the less severe nonsyndromic predispositions with (FH) or without (noFH) Family History [73]. The spectrum of P53 germline mutations comprises many different alleles but the vast majority yields single amino-acid changes in the DBD of the protein [96]. The wide variety of adult-onset and childhood cancers and the distribution of P53 mutations were deeply examined, allowing an understanding of cancer

genotype-phenotype correlations. Nevertheless, the LFS phenotype is complex and cannot be readily explained by the simple identification of P53 germline mutations in affected individuals [73].

Using available data in a yeast-based system, we explored genotype-phenotype correlations by comparing a functional classification (based on transactivation properties) of all P53 germline mutant alleles with clinical data from the IARC database (http://www-p53.iarc.fr/Germline.html) (Figure 2, A2). Our analyses revealed that SD alleles were associated with more severe cancer proneness syndromes (e.g. LFS), while PD alleles were associated with less severe cancer proneness conditions (e.g. FH), indicating that the loss of transactivation ability influences clinical manifestations in patients who inherited P53 mutations and developed cancer [82].

Moreover, given the heterozygous state of germline P53 mutations, the existence of a correlation between dominant-negative features and clinical manifestations in patients who inherited mutations and developed cancer was also explored [83]. While a classification based on transactivation alone (determined by the yeast-based quantitative assay) was confirmed to identify familial cancer cases with more severe clinical features, classification based on dominant-negative effects highlighted similar associations but did not reveal distinct clinical subclasses of SD alleles. We concluded that, in carriers of germline P53 mutations the yeast transactivation-based classification of P53 alleles appears more important for genotype-phenotype correlations than dominant-negative effects, and that haplo-insufficiency of the *TP53* gene is an important factor in cancer proneness in humans [83] (Table 2).

All together, these results highlighted the importance of yeast-based functional assays in order to identify clinical correlations, providing also perspectives in the treatments of patients affected by P53-related disorders.

Application of yeast functional assay to mutational spectrometry: hunting for the culprit fingerprints.

The assumption of molecular epidemiology that carcinogens leave fingerprints has suggested that the analysis of the frequency, type and site of mutations in genes frequently altered in cancer may provide clues to the identification of the factors contributing to carcinogenesis. A mutation

spectrum is described by three parameters: (a) where, along the coding sequence, the mutations occur; (b) which type of base pair substitution occurs; and (c) how many times these mutations have been independently observed. From the biological point of view, a mutation spectrum results from a very complex process that takes into account different factors [28]. Specifically, the probability that a certain nucleotide position of a gene is found mutated in a tumor depends on the product of different probabilities, namely that (a) the position is damaged (b) the resulting lesion is not repaired and (c) is misread by the DNA polymerase, causing the fixation of the mutation, and, finally, (d) the mutation is selected in the carcinogenic process due to some growth advantage. All these probabilities are also influenced by the DNA sequence context and by the differences in biological function of the target gene product.

As an example, the relationship between the presence of specific P53 mutations in tumors and the exposure to a carcinogen has been extensively described in the case of skin cancers and sun light exposure; in fact the International Agency for Research on Cancer in 1992 classified the UV radiation from sunlight as a human carcinogen [37]. Upon reaching the skin, UV photons produced DNA damages [16] known as cyclobutane pyrimidine dimers and pyrimidine 6-4 pyrimidone photoproducts. Incorrect repair of these lesions leads to the fixation of specific mutations in epidermal cells (C→T and CC→TT transition mutations at di-pyrimidine sites), contributing to the development of the tumorigenic process [10]. In fact, about 50% of skin cancers exhibit P53 mutations that are a signature of UV-induced mutagenesis (C→T and CC→TT) [6].

Taking advantage of the previously described yeast P53 functional assay, we adapted it as a new tool for molecular epidemiology [28]. In this assay, a yeast expression vector containing the cDNA of human *TP53* gene was damaged *in vitro* with a specific mutagen/carcinogen (e.g. UV) and transfected in the yeast reporter strain yIG397, exploiting the *ADE2* reporter gene under the control of a P53 RE. Mutant P53s (induced by the DNA damage following the *in vitro* treatment) were identified on a functional basis. In fact, by selecting on plates with limiting adenine, colonies of yeast transformants expressing a P53 protein able or not to transactivate the *ADE2* reporter gene were white or red, respectively. With this approach, it was shown that the yeast functional assay could be used to determine highly specific mutation fingerprints in the human P53 cDNA sequence [28, 39]. In particular, the mutational spectrum induced by UV in the yeast P53 functional assay was indistinguishable from the one observed in non-melanoma

skin cancers at the *TP53* locus, confirming that P53 carcinogen fingerprint obtained in yeast may support the etiology of P53 mutation spectra in specific human tumors (Table 2) (Figure 2, B2).

B. P63 and P73

P63 and P73 proteins belong to the P53 family of TFs. They share a N-terminal transactivation domain, a central sequence specific DBD and an oligomerization domain within the C-terminus. An additional C-terminal domain (SAM domain), probably involved in protein-protein interactions, is present in P63 and P73 proteins. The three proteins have also a common gene structure that produces two groups of mRNAs encoding proteins with different N-terminal regions (ΔN and TA). Moreover, all P53 family transcripts are subjected to alternative splicing mechanisms at C-terminus, generating a variety of isoforms [8, 17]. As P53, P63 and P73 can influence a plethora of cellular pathways [88, 97] mainly through direct binding to sequence specific REs whose consensus sequence is similar to that of p53 REs.

Despite these similarities, the overlap in cellular functions between P53, P63 and P73 is limited as highlighted by comparing their role in human physiology. In fact, unlike P53, P63 and P73 are rarely mutated in cancer and their expression is often deregulated through a variety of mechanisms including epigenetic silencing (e.g. *TP73* gene), imbalanced expression of different N-terminal isoforms and formation of complexes with inhibitory proteins such as mutant P53 [1, 26, 44]. Moreover, P53 germline mutations are associated with the development of the cancer-prone LFS or LFL syndromes [74]. No genetic disorder has been linked to P73 germinal mutations, whereas heterozygous mutations in the *TP63* gene underlie a subset of human ectodermal dysplasia syndromes [105]. The limited overlap in cellular functions among P53 family members also emerged by studying the phenotype of corresponding knockout mice [22, 151, 152].

The transactivation potential of P63 and P73 proteins has been investigated in the yeast-based system exploiting both a *HIS3* and a *GFP* reporter. For example, Kato *et al.*, [56] examined the effect of four tumor derived P63 missense mutations (Q31H, S145L, A148P, Q165L) and seven P63 missense mutations (R204H, R204L, G276V, R279Q, R280S, R304H and R313W) that correspond to P53 hotspot mutations (R175H, R175L, G245V, R248Q, R249S, R273H and R282W) using a transcription assay based on a *GFP* reporter plasmid. While most of

P63 missense mutations at the P53 hotspot residues were transcriptionally inactive, the tumor-derived P63 missense mutations retained the ability to transactivate some promoters. It is important to remember that, in contrast to P53, only few P63 missense mutations have been observed in tumors, restricting their pathogenic relevance in cancer progression.

Shimada *et al.*, [116] studied the ability of wild-type P63 to activate a variety of P53-responsive promoter (p21/WAF1, BAX, MDM2 and 14-3-3σ) in *S. cerevisiae* using both *HIS3* and *GFP* reporter. From all these studies, it emerged that P63 (in particular the γ isoform) has distinct transactivation ability in relation to the different target promoter but also with respect to the activity of wild-type P53 in a temperature-dependent manner (Table 2). The rapid and qualitative yeast assay based on *HIS3* reporter was also used in order to evaluate the transcriptional activity of P73α and P73β isoforms [20], reporting comparable transactivation ability. Our group studied the functional properties of P73β isoform exploiting the *ADE2* color (red-white) reporter and also evaluated the potential for mutant P53s to inhibit P73β activity as a mechanism of gain of function (GOF) of mutants P53s [82].

The issue of P53 family transactivation specificity was deeply investigated by our group taking advantage of the previously described yeast functional assay (luciferase reporter-based assay) [15] (Table 2). The same assay highlighted also the functional heterogeneity of P63 germline mutations associated to the development of human Ectodermal Dysplasia Syndromes [84] (Supplementary Table 3). Interestingly, the observations made in yeast were confirmed in a mammalian cell-based system, supporting the high versatility of the yeast-based functional assay in support of basic as well as clinical research [81, 84].

C. NF-κB

NF-κB proteins are central players of pro-inflammatory and innate immunity responses [92]. The NF-κB family comprises five subunits *NFKB1* (p50), *NFKB2* (p52), *RELA* (p65), C-Rel, and *RELB,* which form either homo or hetero-dimers [30, 62]. NF-κB proteins are usually constitutively expressed in cells but exist as an inactive complex kept in the cytoplasm by the interaction with the inhibitory cofactor IκB. Such inhibition is relieved by phosphorylation of IκB by the IKK complex (IκB kinase) in response to specific stimuli, such as the exposure of cells to inflammatory cytokines, like TNFα (Tumor Necrosis Factor α), or to molecules

associated with pathogen infections (PAMPS, Pathogen Associated Molecular Patterns). Interactions of these extracellular factors with their receptors (TNFRSF or TLRs) [63] activate signal transduction pathways, leading to IκB phosphorylation and degradation, enabling NF-κB proteins to relocate to the nucleus acting as a sequence-specific TF [46, 54, 95, 112, 118, 147]. NF-κB, as homo- or hetero-dimer, recognizes a 10 nucleotides consensus RE (5'-GGGRNWYYCC-3'). Various homo- or hetero- dimers are reported to act in cellular context although the p50/p65 complex is the most active and abundant. p50 and p52 proteins lack a transactivation domain and hence cannot function as homodimers without additional cofactors like BCL3 or BCL6 to activate target genes [29, 110]. ChIP-sequencing data suggest that more than 1000 genes can be regulated by NF-κB proteins, including chemokines, immunoreceptors, stress response genes, regulators of apoptosis and growth factors. Approximately 1.5 x 10^5 NF-kB binding sites have been mapped in the human genome [90]. Moreover, various non-traditional dimer-specific NF-κB binding sites are identified through protein binding microarrays and surface Plasmon resonance techniques without any knowledge of their transactivation abilities [128, 148]. The interplay between the NF-κB family members leads to early or late responsiveness of the target genes in genomic context.

Many studies established that even a single nucleotide variation in a NF-κB RE can profoundly alter the transactivation capacity of NF-κB proteins, impacting both on affinity and selectivity of NF-κB protein interactions [66, 138]. The transactivation capacity of a NF-κB protein has positive correlation with the RE binding affinity [35, 144]. Various non-canonical NF-κB binding sites have been identified, using techniques such as gel-shifts, ChIP-sequencing and Selex (Systematic Evolution of Ligands by Exponential Enrichment) specifically focusing on the p65 subunit of NF-κB [119]. The degeneracy in NF-κB consensus RE provides opportunity for complex cis-regulation, transactivation specificity and gene expression tuning [131].

As for the case of P53 family proteins, there are no clear NF-κB homologs in yeast, but the proteins can act as sequence-specific TFs [24]. Furthermore, co-expression of IκB can inhibit p65 transactivation function [24]. In a recent study we have expanded on those original findings, exploiting variable expression of NF-κB proteins, developing a transcription competent p50 chimera and testing up to 17 different REs [113]. The RE panel included examples of NF-κB *in vivo* targets in the *MCP-1*, *IP-10*, and *IFN β* promoters and others chosen in order to sample

single nucleotide variations in the binding sites resulting in differences in binding affinity. A comparison between reporters with a single NF-κB-RE (decamer), and two repeats (two decamers), either adjacent or spaced, was elucidated in this report (Table 2).

D. Other classes of mammalian TFs tested in yeast to investigate transactivation specificity and the impact of mutations

The *ADE2* (red/white) or the luciferase assay in yeast has also been adapted to study the homeodomain protein NKX2.5 [41] and the bHLH (basic helix-loop-helix) proteins HAND1, E12 and E47 [102]. The bHLH proteins are important regulators of embryonic development, particularly in neurogenesis, heart development, myogenesis and hematopoiesis.

In the case of HAND1, it was possible to study the sequence specificity of E12/E47 homodimers and the impact of co-expression of HAND1, which led to a reduced transactivation from a reporter containing an E-box RE but increased transactivation of the same reporter system containing instead a D-box as upstream RE. The assays proved sensitive also to analyse missense changes both in NKX2.5 and HAND1, revealing both complete and partial loss of transactivation function or, for HAND1, loss of the capacity to modulate E12/E47 transactivation function. For these latter group of proteins, the inclusion of a chimeric transactivation domain increased or revealed the transactivation potential, confirming previous observation that not all type of mammalian transactivation domains are full active with the yeast basal transcription machinery [57].

c-MYC, a pleiotropic TF and a potent oncogene, is another bHLH protein that was shown to act as a TF when expressed in yeast [3] and whose protein stability appeared to be affected by conserved regulatory post-translational modifications [25].

Moreover, a yeast transcription assay with human β-catenin/TCF (T-cell Transcription Factor) complex was developed that could provide for a functional diagnosis of mutations in APC, that can interact with β-catenin leading to its degradation [150]. This latter assay is a clear example of how complex functional interactions between human TFs and protein partners can be recapitulated in yeast cells.

A growth assay was developed to test small molecules affecting the function of HSF1, a TF regulating the expression of heat shock protein genes [91]. In this case, an orthologous,

essential gene is present in the yeast genome; hence, the endogenous gene was placed under a repressible promoter making the growth of yeast dependent on human HSF1 expression and potentially impacted by the treatment with small molecule modulators of human HSF1 [91].

Drugging mammalian TFs in yeast

The yeast systems described above represent well-defined assays with the potential to evaluate or screen small molecules targeting specific TFs and acting either as agonist or antagonist. Different from yeast one- two- or three-hybrid assays [18, 47, 103, 139], no heterologous domains for transactivation, DNA binding nor dual-function small molecule tethers are needed. Further, unlike the case in mammalian cells, these yeast-based functional assays would enable the test of separate members of complex TF families, or the co-expression of specific protein cofactors. However, chemicals uptake or extrusion has been considered a limiting factor in using yeast for drug screening, despite the availability of specific mutants in the ABC transporter systems that showed improved efficacy of various types of small molecules [128]. There are however, examples where small molecules could be assayed for P53, P73, or NF-κB (Table 2) [4, 31, 64, 124, 125, 134].

The capacity of several nuclear receptors to act as ligand-dependent sequence-specific TFs when expressed in yeast was established, among others, for the ER, the Progesterone Receptor (PR), the co-expression of Aromatase and Androgen Receptor (AR), and the Glucocorticoid Receptor (GR) [33, 50, 60, 72, 77, 98, 100, 148]. Assays were also developed for Retinoic acid, Peroxisome Proliferator-Activated Receptor, and Retinoid X Receptor [32, 34, 115]. The crosstalk of nuclear receptors with co-activator and co-repressors has also been explored [137]. Those findings led to the development of yeast assays for the screening of agonist or antagonist molecules [70], adapted to high-throughput format [7, 142]. Although functional analogs of nuclear receptor may exist [89], yeast does not express ER, PR or GR homologs; therefor the study of ligand molecules in this heterologous system could benefit from the lack of the interference from possible hormonal cross talk.

Conclusions and Perspectives

Yeast has been, and remains, a robust model that, even in the era of CRISPR/Cas9 genome editing [21, 48], can be turned into a sort of *in vivo test-tube* for the functional analysis of mammalian sequence-specific TFs. In fact, combining inducible promoter systems, quantitative reporter genes integrated at desired chromosomal locus, small volume format, and the potential to acquire transcription output also at single cell level, this heterologous approach can contribute to elucidate specific aspects of TF function, particularly in assessing the impact of inherited or acquired genetic variability at the level of TF interaction with DNA binding sites, through a transcriptional readout. The system is customizable with relatively ease and rapidity, and can also be scaled-up for cost-effective high-throughput screening, providing a matrix of readout data where, level of TF protein, kind of DNA target site, and time from induction of TF expression can be controlled as distinct variables, comparing isogenic panels of reporter strains, an approach akin to *in vivo* biochemistry. For these features yeast functional assays can still be superior to reporter assays in mammalian cells and could be considered as complementary tools to *in vivo* analyses in many contexts.

Acknowledgments

This work was partially supported by Compagnia S. Paolo, Turin, Italy (Project 2012.1590 *"Le interazioni molecolari della proteina p53 mutata come bersaglio di nuove terapie antitumorali personalizzate"* to GF) and also by the Italian Ministry of Health, 5 per mille 2013 (to GF) and Current Research 2016,by the Italian Association for Cancer Research, AIRC (IG#5506 to G.F.).

Conflict of Interests

The Authors declare that there is no conflict of interest.

Lists of abbreviations

AR, Androgen Receptor; **bHLH,** basic helix-loop-helix; **BS,** Binding Site; **DBD,** DNA Binding Domain; **FH,** Family History; **GOF**, gain of function; **GR**, Glucocorticoid Receptor; **LFL,** Li-

Fraumeni like Syndrome**; LFS,** Li-Fraumeni Syndrome; **noFH,** no Family History; **PD,** Partial Defective; **PR**, Progesterone Receptor, **RE,** Response Element; **SD,** Severe Defective; **SELEX**, Systematic Evolution of Ligands by Exponential Enrichment; **TFs,** Transcription Factor(s).

References

1. Alexandrova, E.M. and U.M. Moll, *Role of p53 family members p73 and p63 in human hematological malignancies.* Leuk Lymphoma, 2012. **53**(11): p. 2116-29.
2. Allen, M.A., Z. Andrysik, V.L. Dengler, H.S. Mellert, A. Guarnieri, J.A. Freeman, K.D. Sullivan, M.D. Galbraith, X. Luo, W.L. Kraus, R.D. Dowell, and J.M. Espinosa, *Global analysis of p53-regulated transcription identifies its direct targets and unexpected regulatory mechanisms.* Elife, 2014. **3**: p. e02200.
3. Amati, B., S. Dalton, M.W. Brooks, T.D. Littlewood, G.I. Evan, and H. Land, *Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max.* Nature, 1992. **359**(6394): p. 423-6.
4. Andreotti, V., Y. Ciribilli, P. Monti, A. Bisio, M. Lion, J. Jordan, G. Fronza, P. Menichini, M.A. Resnick, and A. Inga, *p53 transactivation and the impact of mutations, cofactors and small molecules using a simplified yeast-based screening system.* PLoS One, 2011. **6**(6): p. e20643.
5. Belli, G., E. Gari, L. Piedrafita, M. Aldea, and E. Herrero, *An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast.* Nucleic Acids Res, 1998. **26**(4): p. 942-7.
6. Benjamin, C.L., V.O. Melnikova, and H.N. Ananthaswamy, *P53 protein and pathogenesis of melanoma and nonmelanoma skin cancer.* Adv Exp Med Biol, 2008. **624**: p. 265-82.
7. Berg, M., K. Undisz, R. Thiericke, T. Moore, and C. Posten, *Miniaturization of a functional transcription assay in yeast (human progesterone receptor) in the 384- and 1536-well plate format.* J Biomol Screen, 2000. **5**(2): p. 71-6.
8. Bourdon, J.C., *p53 Family isoforms.* Curr Pharm Biotechnol, 2007. **8**(6): p. 332-6.
9. Brachmann, R.K., K. Yu, Y. Eby, N.P. Pavletich, and J.D. Boeke, *Genetic selection of intragenic suppressor mutations that reverse the effect of common p53 cancer mutations.* Embo J, 1998. **17**(7): p. 1847-59.
10. Cadet, J., E. Sage, and T. Douki, *Ultraviolet radiation-mediated damage to cellular DNA.* Mutat Res, 2005. **571**(1-2): p. 3-17.
11. Campomenosi, P., P. Monti, A. Aprile, A. Abbondandolo, T. Frebourg, B. Gold, T. Crook, A. Inga, M.A. Resnick, R. Iggo, and G. Fronza, *p53 mutants can often transactivate promoters containing a p21 but not Bax or PIG3 responsive elements.* Oncogene, 2001. **20**(27): p. 3573-9.

12. Chappuis, P.O., A. Estreicher, B. Dieterich, H. Bonnefoi, M. Otter, A.P. Sappino, and R. Iggo, *Prognostic significance of p53 mutation in breast cancer: frequent detection of non-missense mutations by yeast functional assay.* Int J Cancer, 1999. **84**(6): p. 587-93.
13. Cho, Y., S. Gorina, P.D. Jeffrey, and N.P. Pavletich, *Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations [see comments]*. Science, 1994. **265**(5170): p. 346-55.
14. Ciribilli, Y., V. Andreotti, D. Menendez, J.S. Langen, G. Schoenfelder, M.A. Resnick, and A. Inga, *The coordinated p53 and estrogen receptor cis-regulation at an FLT1 promoter SNP is specific to genotoxic stress and estrogenic compound.* PLoS One, 2010. **5**(4): p. e10236.
15. Ciribilli, Y., P. Monti, A. Bisio, H.T. Nguyen, A.S. Ethayathulla, A. Ramos, G. Foggetti, P. Menichini, D. Menendez, M.A. Resnick, H. Viadiu, G. Fronza, and A. Inga, *Transactivation specificity is conserved among p53 family proteins and depends on a response element sequence code.* Nucleic Acids Res, 2013. **41**(18): p. 8637-53.
16. Clingen, P.H., C.F. Arlett, L. Roza, T. Mori, O. Nikaido, and M.H. Green, *Induction of cyclobutane pyrimidine dimers, pyrimidine(6-4)pyrimidone photoproducts, and Dewar valence isomers by natural sunlight in normal human mononuclear cells.* Cancer Res, 1995. **55**(11): p. 2245-8.
17. Collavin, L., A. Lunardi, and G. Del Sal, *p53-family proteins and their regulators: hubs and spokes in tumor suppression.* Cell Death Differ, 2010. **17**(6): p. 901-11.
18. Cottier, S., T. Monig, Z. Wang, J. Svoboda, W. Boland, M. Kaiser, and E. Kombrink, *The yeast three-hybrid system as an experimental platform to identify proteins interacting with small signaling molecules in plant cells: potential and limitations.* Front Plant Sci, 2011. **2**: p. 101.
19. Das, M.K. and H.K. Dai, *A survey of DNA motif finding algorithms.* BMC Bioinformatics, 2007. **8 Suppl 7**: p. S21.
20. Di Como, C.J., C. Gaiddon, and C. Prives, *p73 function is inhibited by tumor-derived p53 mutants in mammalian cells.* Mol Cell Biol, 1999. **19**(2): p. 1438-49.
21. DiCarlo, J.E., J.E. Norville, P. Mali, X. Rios, J. Aach, and G.M. Church, *Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems.* Nucleic Acids Res, 2013. **41**(7): p. 4336-43.
22. Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery, Jr., J.S. Butel, and A. Bradley, *Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours.* Nature, 1992. **356**(6366): p. 215-21.
23. el-Deiry, W.S., S.E. Kern, J.A. Pietenpol, K.W. Kinzler, and B. Vogelstein, *Definition of a consensus binding site for p53.* Nat Genet, 1992. **1**(1): p. 45-9.
24. Epinat, J.C., S.T. Whiteside, N.R. Rice, A. Israel, T.D. Gilmore, K.M. Ryan, M.K. Ernst, K.H. Vousden, D.B. Huang, D. Vu, L.A. Cassiday, J.M. Zimmerman, L.J. Maher, 3rd, and G. Ghosh, *Reconstitution of the NF-kappa B system in Saccharomyces cerevisiae.* Yeast, 1997. **13**(7): p. 599-612.
25. Escamilla-Powers, J.R. and R.C. Sears, *A conserved pathway that controls c-Myc protein stability through opposing phosphorylation events occurs in yeast.* J Biol Chem, 2007. **282**(8): p. 5432-42.
26. Ferraiuolo, M., S. Di Agostino, G. Blandino, and S. Strano, *Oncogenic Intra-p53 Family Member Interactions in Human Cancers.* Front Oncol, 2016. **6**: p. 77.

27. Flaman, J.M., T. Frebourg, V. Moreau, F. Charbonnier, C. Martin, P. Chappuis, A.P. Sappino, I.M. Limacher, L. Bron, J. Benhattar, and et al., *A simple p53 functional assay for screening cell lines, blood, and tumors.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3963-7.
28. Fronza, G., A. Inga, P. Monti, G. Scott, P. Campomenosi, P. Menichini, L. Ottaggio, S. Viaggi, P.A. Burns, B. Gold, and A. Abbondandolo, *The yeast p53 functional assay: a new tool for molecular epidemiology. Hopes and facts.* Mutat Res, 2000. **462**(2-3): p. 293-301.
29. Ghosh, G., G. van Duyne, S. Ghosh, and P.B. Sigler, *Structure of NF-kappa B p50 homodimer bound to a kappa B site.* Nature, 1995. **373**(6512): p. 303-10.
30. Ghosh, S., M.J. May, and E.B. Kopp, *NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses.* Annu Rev Immunol, 1998. **16**: p. 225-60.
31. Gomes, S., M. Leao, L. Raimundo, H. Ramos, J. Soares, and L. Saraiva, *p53 family interactions and yeast: together in anticancer therapy.* Drug Discov Today, 2016. **21**(4): p. 616-24.
32. Heery, D.M., T. Zacharewski, B. Pierrat, H. Gronemeyer, P. Chambon, and R. Losson, *Efficient transactivation by retinoic acid receptors in yeast requires retinoid X receptors.* Proc Natl Acad Sci U S A, 1993. **90**(9): p. 4281-5.
33. Heldring, N., A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, M. Tujague, A. Strom, E. Treuter, M. Warner, and J.A. Gustafsson, *Estrogen receptors: how do they signal and what are their targets.* Physiol Rev, 2007. **87**(3): p. 905-31.
34. Henry, K., M.L. O'Brien, W. Clevenger, L. Jow, and D.J. Noonan, *Peroxisome proliferator-activated receptor response specificities as defined in yeast and mammalian cell transcription assays.* Toxicol Appl Pharmacol, 1995. **132**(2): p. 317-24.
35. Hoffmann, A., T.H. Leung, and D. Baltimore, *Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities.* Embo j, 2003. **22**(20): p. 5530-9.
36. Horvath, M.M., X. Wang, M.A. Resnick, and D.A. Bell, *Divergent evolution of human p53 binding sites: cell cycle versus apoptosis.* PLoS Genet, 2007. **3**(7): p. e127.
37. IARC, *Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, ed. L. International Agency for Research on Cancer, France. Vol. 55. 1992.
38. Iggo, R., J. Rudewicz, E. Monceau, N. Sevenet, J. Bergh, T. Sjoblom, and H. Bonnefoi, *Validation of a yeast functional assay for p53 mutations using clonal sequencing.* J Pathol, 2013. **231**(4): p. 441-8.
39. Inga, A., R. Iannone, P. Monti, F. Molina, M. Bolognesi, A. Abbondandolo, R. Iggo, and G. Fronza, *Determining mutational fingerprints at the human p53 locus with a yeast functional assay: a new tool for molecular epidemiology.* Oncogene, 1997. **14**(11): p. 1307-13.
40. Inga, A., P. Monti, G. Fronza, T. Darden, and M.A. Resnick, *p53 mutants exhibiting enhanced transcriptional activation and altered promoter selectivity are revealed using a sensitive, yeast-based functional assay.* Oncogene, 2001. **20**(4): p. 501-13.
41. Inga, A., S.M. Reamon-Buettner, J. Borlak, and M.A. Resnick, *Functional dissection of sequence-specific NKX2-5 DNA binding domain mutations associated with human heart septation defects using a yeast-based system.* Hum Mol Genet, 2005. **14**(14): p. 1965-75.
42. Inga, A. and M.A. Resnick, *Novel human p53 mutations that are toxic to yeast can enhance transactivation of specific promoters and reactivate tumor p53 mutants.* Oncogene, 2001. **20**(26): p. 3409-19.

43. Inga, A., F. Storici, T.A. Darden, and M.A. Resnick, *Differential transactivation by the p53 transcription factor is highly dependent on p53 level and promoter target sequence.* Mol Cell Biol, 2002. **22**(24): p. 8612-25.
44. Inoue, K. and E.A. Fry, *Alterations of p63 and p73 in human cancers.* Subcell Biochem, 2014. **85**: p. 17-40.
45. Ishioka, C., T. Frebourg, Y.X. Yan, M. Vidal, S.H. Friend, S. Schmidt, and R. Iggo, *Screening patients for heterozygous p53 mutations using a functional assay in yeast.* Nat Genet, 1993. **5**(2): p. 124-9.
46. Jacobs, M.D. and S.C. Harrison, *Structure of an IkappaBalpha/NF-kappaB complex.* Cell, 1998. **95**(6): p. 749-58.
47. Jaeger, S., G. Eriani, and F. Martin, *Results and prospects of the yeast three-hybrid system.* FEBS Lett, 2004. **556**(1-3): p. 7-12.
48. Jakociunas, T., I. Bonde, M. Herrgard, S.J. Harrison, M. Kristensen, L.E. Pedersen, M.K. Jensen, and J.D. Keasling, *Multiplex metabolic pathway engineering using CRISPR/Cas9 in Saccharomyces cerevisiae.* Metab Eng, 2015. **28**: p. 213-22.
49. Jegga, A.G., A. Inga, D. Menendez, B.J. Aronow, and M.A. Resnick, *Functional evolution of the p53 regulatory network through its target response elements.* Proc Natl Acad Sci U S A, 2008. **105**(3): p. 944-9.
50. Jin, L., D.Q. Tran, C.F. Ide, J.A. McLachlan, and S.F. Arnold, *Several synthetic chemicals inhibit progesterone receptor-mediated transactivation in yeast.* Biochem Biophys Res Commun, 1997. **233**(1): p. 139-46.
51. Jordan, J.J., A. Inga, K. Conway, S. Edmiston, L.A. Carey, L. Wu, and M.A. Resnick, *Altered-function p53 missense mutations identified in breast cancers can have subtle effects on transactivation.* Mol Cancer Res, 2010. **8**(5): p. 701-16.
52. Jordan, J.J., D. Menendez, J. Sharav, I. Beno, K. Rosenthal, M.A. Resnick, and T.E. Haran, *Low-level p53 expression changes transactivation rules and reveals superactivating sequences.* Proc Natl Acad Sci U S A, 2012. **109**(36): p. 14387-92.
53. Kaeberlein, M., C.R. Burtner, and B.K. Kennedy, *Recent Developments in Yeast Aging.* PLoS Genet, 2007. **3**(5): p. e84.
54. Karin, M., *How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex.* Oncogene, 1999. **18**(49): p. 6867-74.
55. Kato, S., S.Y. Han, W. Liu, K. Otsuka, H. Shibata, R. Kanamaru, and C. Ishioka, *Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8424-9.
56. Kato, S., A. Shimada, M. Osada, S. Ikawa, M. Obinata, A. Nakagawara, R. Kanamaru, and C. Ishioka, *Effects of p51/p63 missense mutations on transcriptional activities of p53 downstream gene promoters.* Cancer Res, 1999. **59**(23): p. 5908-11.
57. Kennedy, B.K., *Mammalian transcription factors in yeast: strangers in a familiar land.* Nat Rev Mol Cell Biol, 2002. **3**(1): p. 41-9.
58. Kitayner, M., H. Rozenberg, N. Kessler, D. Rabinovich, L. Shaulov, T.E. Haran, and Z. Shakked, *Structural basis of DNA recognition by p53 tetramers.* Mol Cell, 2006. **22**(6): p. 741-53.
59. Kitayner, M., H. Rozenberg, R. Rohs, O. Suad, D. Rabinovich, B. Honig, and Z. Shakked, *Diversity in DNA recognition by p53 revealed by crystal structures with Hoogsteen base pairs.* Nat Struct Mol Biol, 2010. **17**(4): p. 423-9.

60. Kolle, S.N., H.G. Kamp, H.A. Huener, J. Knickel, A. Verlohner, C. Woitkowiak, R. Landsiedel, and B. van Ravenzwaay, *In house validation of recombinant yeast estrogen and androgen receptor agonist and antagonist screening assays.* Toxicol In Vitro, 2010. **24**(7): p. 2030-40.
61. Kovvali, G.K., B. Mehta, C.B. Epstein, and S.G. Lutzker, *Identification of partial loss of function p53 gene mutations utilizing a yeast-based functional assay.* Nucleic Acids Res, 2001. **29**(5): p. E28.
62. Kunsch, C., S.M. Ruben, and C.A. Rosen, *Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation.* Mol Cell Biol, 1992. **12**(10): p. 4412-21.
63. Lawrence, T., *The Nuclear Factor NF-κB Pathway in Inflammation.* Cold Spring Harb Perspect Biol, 2009. **1**(6).
64. Leao, M., S. Gomes, J. Soares, C. Bessa, C. Maciel, Y. Ciribilli, C. Pereira, A. Inga, and L. Saraiva, *Novel simplified yeast-based assays of regulators of p53-MDMX interaction and p53 transcriptional activity.* FEBS J, 2013. **280**(24): p. 6498-507.
65. Leroy, B., J.L. Fournier, C. Ishioka, P. Monti, A. Inga, G. Fronza, and T. Soussi, *The TP53 website: an integrative resource centre for the TP53 mutation database and TP53 mutant analysis.* Nucleic Acids Res, 2013. **41**(Database issue): p. D962-9.
66. Leung, T.H., A. Hoffmann, and D. Baltimore, *One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers.* Cell, 2004. **118**(4): p. 453-64.
67. Lion, M., I. Raimondi, S. Donati, O. Jousson, Y. Ciribilli, and A. Inga, *Evolution of p53 transactivation specificity through the lens of a yeast-based functional assay.* PLoS One, 2015. **10**(2): p. e0116177.
68. Lozano, G., *Mouse models of p53 functions.* Cold Spring Harb Perspect Biol, 2010. **2**(4): p. a001115.
69. Ludwig, R.L., S. Bates, and K.H. Vousden, *Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function.* Mol Cell Biol, 1996. **16**(9): p. 4952-60.
70. Lyttle, C.R., P. Damian-Matsumura, H. Juul, and T.R. Butt, *Human estrogen receptor regulation in a yeast model system and studies on receptor agonists and antagonists.* J Steroid Biochem Mol Biol, 1992. **42**(7): p. 677-85.
71. Mahony, S. and B.F. Pugh, *Protein-DNA binding in high-resolution.* Crit Rev Biochem Mol Biol, 2015. **50**(4): p. 269-83.
72. Mak, P., F.D. Cruz, and S. Chen, *A yeast screen system for aromatase inhibitors and ligands for androgen receptor: yeast cells transformed with aromatase and androgen receptor.* Environ Health Perspect, 1999. **107**(11): p. 855-60.
73. Malkin, D., *Li-fraumeni syndrome.* Genes Cancer, 2011. **2**(4): p. 475-84.
74. Malkin, D., F.P. Li, L.C. Strong, J.F. Fraumeni, Jr., C.E. Nelson, D.H. Kim, J. Kassel, M.A. Gryka, F.Z. Bischoff, M.A. Tainsky, and et al., *Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms.* Science, 1990. **250**(4985): p. 1233-8.
75. Menendez, D., A. Inga, and M.A. Resnick, *The biological impact of the human master regulator p53 can be altered by mutations that change the spectrum and expression of its target genes.* Mol Cell Biol, 2006. **26**(6): p. 2297-308.
76. Menendez, D., A. Inga, and M.A. Resnick, *The expanding universe of p53 targets.* Nat Rev Cancer, 2009. **9**(10): p. 724-37.

77. Menendez, D., A. Inga, and M.A. Resnick, *Estrogen receptor acting in cis enhances WT and mutant p53 transactivation at canonical and noncanonical p53 target sequences.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1500-5.
78. Menendez, D., A. Inga, and M.A. Resnick, *Potentiating the p53 network.* Discov Med, 2010. **10**(50): p. 94-100.
79. Monti, P., P. Campomenosi, Y. Ciribilli, R. Iannone, A. Aprile, A. Inga, M. Tada, P. Menichini, A. Abbondandolo, and G. Fronza, *Characterization of the p53 mutants ability to inhibit p73 beta transactivation using a yeast-based functional assay.* Oncogene, 2003. **22**(34): p. 5252-60.
80. Monti, P., P. Campomenosi, Y. Ciribilli, R. Iannone, A. Inga, A. Abbondandolo, M.A. Resnick, and G. Fronza, *Tumour p53 mutations exhibit promoter selective dominance over wild type p53.* Oncogene, 2002. **21**(11): p. 1641-8.
81. Monti, P., Y. Ciribilli, A. Bisio, G. Foggetti, I. Raimondi, P. Campomenosi, P. Menichini, G. Fronza, and A. Inga, *N-P63alpha and TA-P63alpha exhibit intrinsic differences in transactivation specificities that depend on distinct features of DNA target sites.* Oncotarget, 2014. **5**(8): p. 2116-30.
82. Monti, P., Y. Ciribilli, J. Jordan, P. Menichini, D.M. Umbach, M.A. Resnick, L. Luzzatto, A. Inga, and G. Fronza, *Transcriptional functionality of germ line p53 mutants influences cancer phenotype.* Clin Cancer Res, 2007. **13**(13): p. 3789-95.
83. Monti, P., C. Perfumo, A. Bisio, Y. Ciribilli, P. Menichini, D. Russo, D.M. Umbach, M.A. Resnick, A. Inga, and G. Fronza, *Dominant-negative features of mutant TP53 in germline carriers have limited impact on cancer outcomes.* Mol Cancer Res, 2011. **9**(3): p. 271-9.
84. Monti, P., D. Russo, R. Bocciardi, G. Foggetti, P. Menichini, M.T. Divizia, M. Lerone, C. Graziano, A. Wischmeijer, H. Viadiu, R. Ravazzolo, A. Inga, and G. Fronza, *EEC- and ADULT-associated TP63 mutations exhibit functional heterogeneity toward P63 responsive sequences.* Hum Mutat, 2013. **34**(6): p. 894-904.
85. Muller, P.A., K.H. Vousden, and J.C. Norman, *p53 and its mutants in tumor cell migration and invasion.* J Cell Biol, 2011. **192**(2): p. 209-18.
86. Mumberg, D., R. Muller, and M. Funk, *Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression.* Nucleic Acids Res, 1994. **22**(25): p. 5767-8.
87. Mundade, R., H.G. Ozer, H. Wei, L. Prabhu, and T. Lu, *Role of ChIP-seq in the discovery of transcription factor binding sites, differential gene regulation mechanism, epigenetic marks and beyond.* Cell Cycle, 2014. **13**(18): p. 2847-52.
88. Murray-Zmijewski, F., D.P. Lane, and J.C. Bourdon, *p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress.* Cell Death Differ, 2006. **13**(6): p. 962-72.
89. Naar, A.M. and J.K. Thakur, *Nuclear receptor-like transcription factors in fungi.* Genes Dev, 2009. **23**(4): p. 419-32.
90. Natoli, G., S. Saccani, D. Bosisio, and I. Marazzi, *Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time.* Nat Immunol, 2005. **6**(5): p. 439-45.
91. Neef, D.W., M.L. Turski, and D.J. Thiele, *Modulation of heat shock transcription factor 1 as a therapeutic target for small molecule intervention in neurodegenerative disease.* PLoS Biol, 2010. **8**(1): p. e1000291.

92. Oeckinghaus, A. and S. Ghosh, *The NF-kappaB family of transcription factors and its regulation.* Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a000034.
93. Oren, M. and V. Rotter, *Mutant p53 gain-of-function in cancer.* Cold Spring Harb Perspect Biol, 2010. **2**(2): p. a001107.
94. Pan, Y., C.J. Tsai, B. Ma, and R. Nussinov, *Mechanisms of transcription factor selectivity.* Trends Genet, 2010. **26**(2): p. 75-83.
95. Perkins, N.D., *Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway.* Oncogene, 2006. **25**(51): p. 6717-30.
96. Petitjean, A., E. Mathe, S. Kato, C. Ishioka, S.V. Tavtigian, P. Hainaut, and M. Olivier, *Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database.* Hum Mutat, 2007. **28**(6): p. 622-9.
97. Pflaum, J., S. Schlosser, and M. Muller, *p53 Family and Cellular Stress Responses in Cancer.* Front Oncol, 2014. **4**: p. 285.
98. Pham, T.A., Y.P. Hwung, D. Santiso-Mere, D.P. McDonnell, and B.W. O'Malley, *Ligand-dependent and -independent function of the transactivation regions of the human estrogen receptor in yeast.* Mol Endocrinol, 1992. **6**(7): p. 1043-50.
99. Pierrat, B., D.M. Heery, Y. Lemoine, and R. Losson, *Functional analysis of the human estrogen receptor using a phenotypic transactivation assay in yeast.* Gene, 1992. **119**(2): p. 237-45.
100. Popovic, N., S. Ruzdijic, D.T. Kanazir, A. Niciforovic, M. Adzic, E. Paraskevopoulou, C. Pantelidou, M. Radojcic, C. Demonacos, and M. Krstic-Demonacos, *Site-specific and dose-dependent effects of glucocorticoid receptor phosphorylation in yeast Saccharomyces cerevisiae.* Steroids, 2010. **75**(6): p. 457-65.
101. Pott, S. and J.D. Lieb, *What are super-enhancers?* Nat Genet, 2015. **47**(1): p. 8-12.
102. Reamon-Buettner, S.M., Y. Ciribilli, A. Inga, and J. Borlak, *A loss-of-function mutation in the binding domain of HAND1 predicts hypoplasia of the human hearts.* Hum Mol Genet, 2008. **17**(10): p. 1397-405.
103. Reece-Hoyes, J.S. and A.J. Marian Walhout, *Yeast one-hybrid assays: a historical and technical perspective.* Methods, 2012. **57**(4): p. 441-7.
104. Resnick, M.A. and A. Inga, *Functional mutants of the sequence-specific transcription factor p53 and implications for master genes of diversity.* Proc Natl Acad Sci U S A, 2003. **100**(17): p. 9934-9.
105. Rinne, T., H.G. Brunner, and H. van Bokhoven, *p63-associated disorders.* Cell Cycle, 2007. **6**(3): p. 262-8.
106. Rubbi, C.P. and J. Milner, *p53 is a chromatin accessibility factor for nucleotide excision repair of DNA damage.* EMBO J, 2003. **22**(4): p. 975-86.
107. Saller, E., E. Tom, M. Brunori, M. Otter, A. Estreicher, D.H. Mack, and R. Iggo, *Increased apoptosis induction by 121F mutant p53.* Embo J, 1999. **18**(16): p. 4424-37.
108. Sammons, M.A., J. Zhu, A.M. Drake, and S.L. Berger, *TP53 engagement with the genome occurs in distinct local chromatin environments via pioneer factor activity.* Genome Res, 2015. **25**(2): p. 179-88.
109. Scharer, E. and R. Iggo, *Mammalian p53 can function as a transcription factor in yeast.* Nucleic Acids Res, 1992. **20**(7): p. 1539-45.
110. Schmitz, M.L. and P.A. Baeuerle, *The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B.* Embo j, 1991. **10**(12): p. 3805-17.

111. Sebastian, A. and B. Contreras-Moreira, *The twilight zone of cis element alignments.* Nucleic Acids Res, 2013. **41**(3): p. 1438-49.
112. Sen, R. and D. Baltimore, *Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism.* Cell, 1986. **47**(6): p. 921-8.
113. Sharma, V., J.J. Jordan, Y. Ciribilli, M.A. Resnick, A. Bisio, and A. Inga, *Quantitative Analysis of NF-kappaB Transactivation Specificity Using a Yeast-Based Functional Assay.* PLoS One, 2015. **10**(7): p. e0130170.
114. Shen, S.Q., C.A. Myers, A.E. Hughes, L.C. Byrne, J.G. Flannery, and J.C. Corbo, *Massively parallel cis-regulatory analysis in the mammalian central nervous system.* Genome Res, 2016. **26**(2): p. 238-55.
115. Shiizaki, K., T. Yoshikawa, E. Takada, S. Hirose, S. Ito-Harashima, M. Kawanishi, and T. Yagi, *Development of yeast reporter assay for screening specific ligands of retinoic acid and retinoid X receptor subtypes.* J Pharmacol Toxicol Methods, 2014. **69**(3): p. 245-52.
116. Shimada, A., S. Kato, K. Enjo, M. Osada, Y. Ikawa, K. Kohno, M. Obinata, R. Kanamaru, S. Ikawa, and C. Ishioka, *The transcriptional activities of p53 and its homologue p51/p63: similarities and differences.* Cancer Res, 1999. **59**(12): p. 2781-6.
117. Shiraishi, K., S. Kato, S.Y. Han, W. Liu, K. Otsuka, M. Sakayori, T. Ishida, M. Takeda, R. Kanamaru, N. Ohuchi, and C. Ishioka, *Isolation of temperature-sensitive p53 mutations from a comprehensive missense mutation library.* J Biol Chem, 2004. **279**(1): p. 348-55.
118. Shukla, S., G.T. MacLennan, P. Fu, J. Patel, S.R. Marengo, M.I. Resnick, and S. Gupta, *Nuclear factor-kappaB/p65 (Rel A) is constitutively activated in human prostate adenocarcinoma and correlates with disease progression.* Neoplasia, 2004. **6**(4): p. 390-400.
119. Siggers, T., T.D. Gilmore, B. Barron, and A. Penvose, *Characterizing the DNA binding site specificity of NF-kappaB with protein-binding microarrays (PBMs).* Methods Mol Biol, 2015. **1280**: p. 609-30.
120. Smardova, J., *FASAY: a simple functional assay in yeast for identification of p53 mutation in tumors.* Neoplasma, 1999. **46**(2): p. 80-8.
121. Smardova, J., J. Smarda, and J. Koptikova, *Functional analysis of p53 tumor suppressor in yeast.* Differentiation, 2005. **73**(6): p. 261-77.
122. Smardova, J., V. Vagunda, E. Jandakova, M. Vagundova, H. Koukalova, J. Kovarik, and J. Zaloudik, *p53 status in breast carcinomas revealed by FASAY correlates well with p53 protein accumulation determined by immunohistochemistry.* Neoplasma, 1999. **46**(6): p. 384-9.
123. Smeenk, L., S.J. van Heeringen, M. Koeppel, M.A. van Driel, S.J. Bartels, R.C. Akkers, S. Denissov, H.G. Stunnenberg, and M. Lohrum, *Characterization of genome-wide p53-binding sites upon stress response.* Nucleic Acids Res, 2008. **36**(11): p. 3639-54.
124. Soares, J., N.A. Pereira, A. Monteiro, M. Leao, C. Bessa, D.J. Dos Santos, L. Raimundo, G. Queiroz, A. Bisio, A. Inga, C. Pereira, M.M. Santos, and L. Saraiva, *Oxazoloisoindolinones with in vitro antitumor activity selectively activate a p53-pathway through potential inhibition of the p53-MDM2 interaction.* Eur J Pharm Sci, 2014. **66C**: p. 138-147.
125. Soares, J., L. Raimundo, N.A. Pereira, D.J. dos Santos, M. Perez, G. Queiroz, M. Leao, M.M. Santos, and L. Saraiva, *A tryptophanol-derived oxazolopiperidone lactam is*

cytotoxic against tumors via inhibition of p53 interaction with murine double minute proteins. Pharmacol Res, 2015. **95-96**: p. 42-52.
126. Soussi, T., *TP53 mutations in human cancer: database reassessment and prospects for the next decade.* Adv Cancer Res, 2011. **110**: p. 107-39.
127. Spitz, F. and E.E. Furlong, *Transcription factors: from enhancer binding to developmental control.* Nat Rev Genet, 2012. **13**(9): p. 613-26.
128. Stepanov, A., K.C. Nitiss, G. Neale, and J.L. Nitiss, *Enhancing drug accumulation in Saccharomyces cerevisiae by repression of pleiotropic drug resistance genes with chimeric transcription repressors.* Mol Pharmacol, 2008. **74**(2): p. 423-31.
129. Storici, F., L.K. Lewis, and M.A. Resnick, *In vivo site-directed mutagenesis using oligonucleotides.* Nat Biotechnol, 2001. **19**(8): p. 773-6.
130. Storici, F. and M.A. Resnick, *The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast.* Methods Enzymol, 2006. **409**: p. 329-45.
131. Sullivan, J.C., F.S. Wolenski, A.M. Reitzel, C.E. French, N. Traylor-Knowles, T.D. Gilmore, and J.R. Finnerty, *Two alleles of NF-kappaB in the sea anemone Nematostella vectensis are widely dispersed in nature and encode proteins with distinct activities.* PLoS One, 2009. **4**(10): p. e7311.
132. Sun, S.C., *Non-canonical NF-kappaB signaling pathway.* Cell Res, 2011. **21**(1): p. 71-85.
133. Sunahara, M., T. Shishikura, M. Takahashi, S. Todo, N. Yamamoto, H. Kimura, S. Kato, C. Ishioka, S. Ikawa, Y. Ikawa, and A. Nakagawara, *Mutational analysis of p51A/TAp63gamma, a p53 homolog, in non-small cell lung cancer and breast cancer.* Oncogene, 1999. **18**(25): p. 3761-5.
134. Tanner, S. and A. Barberis, *CP-31398, a putative p53-stabilizing molecule tested in mammalian cells and in yeast for its effects on p53 transcriptional activity.* J Negat Results Biomed, 2004. **3**: p. 5.
135. Tebaldi, T., S. Zaccara, F. Alessandrini, A. Bisio, Y. Ciribilli, and A. Inga, *Whole-genome cartography of p53 response elements ranked on transactivation potential.* BMC Genomics, 2015. **16**: p. 464.
136. Tomso, D.J., A. Inga, D. Menendez, G.S. Pittman, M.R. Campbell, F. Storici, D.A. Bell, and M.A. Resnick, *Functionally distinct polymorphic sequences in the human genome that are targets for p53 transactivation.* Proc Natl Acad Sci U S A, 2005. **102**(18): p. 6431-6.
137. Treuter, E., T. Albrektsen, L. Johansson, J. Leers, and J.A. Gustafsson, *A regulatory role for RIP140 in nuclear receptor activation.* Mol Endocrinol, 1998. **12**(6): p. 864-81.
138. Udalova, I.A., R. Mott, D. Field, and D. Kwiatkowski, *Quantitative prediction of NF-kappa B DNA-protein interactions.* Proc Natl Acad Sci U S A, 2002. **99**(12): p. 8167-72.
139. Van Criekinge, W. and R. Beyaert, *Yeast Two-Hybrid: State of the Art.* Biol Proced Online, 1999. **2**: p. 1-38.
140. Verfaillie, A., D. Svetlichnyy, H. Imrichova, K. Davie, M. Fiers, Z. Kalender Atak, G. Hulselmans, V. Christiaens, and S. Aerts, *Multiplex enhancer-reporter assays uncover unsophisticated TP53 enhancer logic.* Genome Res, 2016.
141. Walerych, D., M. Napoli, L. Collavin, and G. Del Sal, *The rebel angel: mutant p53 as the driving oncogene in breast cancer.* Carcinogenesis, 2012. **33**(11): p. 2007-17.
142. Wang, S. and T.F. Bovee, *Estrogen Receptor Agonists and Antagonists in the Yeast Estrogen Bioassay.* Methods Mol Biol, 2016. **1366**: p. 337-42.

143. Wang, T., J. Zeng, C.B. Lowe, R.G. Sellers, S.R. Salama, M. Yang, S.M. Burgess, R.K. Brachmann, and D. Haussler, *Species-specific endogenous retroviruses shape the transcriptional network of the human tumor suppressor protein p53.* Proc Natl Acad Sci U S A, 2007. **104**(47): p. 18613-8.
144. Wang, V.Y., W. Huang, M. Asagiri, N. Spann, A. Hoffmann, C. Glass, and G. Ghosh, *The transcriptional specificity of NF-kappaB dimers is coded within the kappaB DNA response elements.* Cell Rep, 2012. **2**(4): p. 824-39.
145. Weinhandl, K., M. Winkler, A. Glieder, and A. Camattari, *Carbon source dependent promoters in yeasts.* Microb Cell Fact, 2014. **13**: p. 5.
146. Weir, M. and J.B. Keeney, *PCR mutagenesis and gap repair in yeast.* Methods Mol Biol, 2014. **1205**: p. 29-35.
147. Whiteside, S.T. and A. Israel, *I kappa B proteins: structure, function and regulation.* Semin Cancer Biol, 1997. **8**(2): p. 75-82.
148. Wright, A.P. and J.A. Gustafsson, *Glucocorticoid-specific gene activation by the intact human glucocorticoid receptor expressed in yeast. Glucocorticoid specificity depends on low level receptor expression.* J Biol Chem, 1992. **267**(16): p. 11191-5.
149. Wylie, A., A.E. Jones, A. D'Brot, W.J. Lu, P. Kurtz, J.V. Moran, D. Rakheja, K.S. Chen, R.E. Hammer, S.A. Comerford, J.F. Amatruda, and J.M. Abrams, *p53 genes function to restrain mobile elements.* Genes Dev, 2016. **30**(1): p. 64-77.
150. Yamada, H., K. Furuuchi, T. Aoyama, A. Kataoka, J. Hamada, M. Tada, S. Okushiba, S. Kondo, T. Moriuchi, and H. Katoh, *Reconstructed beta-catenin/TCF4 signaling in yeast applicable to functional evaluation of APC mutations.* Am J Pathol, 2003. **163**(6): p. 2201-9.
151. Yang, A., R. Schweitzer, D. Sun, M. Kaghad, N. Walker, R.T. Bronson, C. Tabin, A. Sharpe, D. Caput, C. Crum, and F. McKeon, *p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development.* Nature, 1999. **398**(6729): p. 714-8.
152. Yang, A., N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, F. McKeon, and D. Caput, *p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours.* Nature, 2000. **404**(6773): p. 99-103.

Table 1. Hallmarks of P53 REs established through yeast-based assays.

Feature / Finding	References
Consensus p53 RE: RRRCWWGYYY N RRRCWWGYYY	23
Nucleotide mismatches in the CWWG core domain, or in the flanking purines (RRR) and pyrimidines (YYY), can greatly alter the RE transactivation potential and specificity, also depending on the expression level of P53 protein	43
Single nucleotide polymorphs (SNPs) can have a significant impact on RE function, causing genetic diversity in the P53 regulatory network	136
A spacer between P53 half-sites, even of one or two nucleotides, can have a strong negative impact on the transactivation potential, depending on its length, the affinity of the P53 protein for RE and the level of P53 protein	76
Non-canonical P53 REs consisting in three-quarter sites (one decamer plus an adjacent half decamer) or in half-sites (a single decamer) can also mediate P53 binding and gene responsiveness	135, 76
Structural properties of DNA can influence P53-dependent transcriptional decisions. For example, a torsionally flexible RE sequence exhibits high transactivation potential even at low P53 levels, apparently due to low dissociation rates of the protein	52
A widespread evolutionary turnover of P53 REs through the period of 500 million years has been captured by a study on comparative analysis of functional *versus* sequence homology of 47 P53 REs found in 38 genes from 14 species	49
Comparing yeast P53 proteins isolated from six chordate species provided an outlook of phenotypic divergence of P53 as a sequence-specific TF	67

Table 2. Main findings from the study on P53, P63 or NF-κB TFs using yeast functional assays

Feature / Finding	References
Mutant P53 alleles are functionally heterogeneous	55; 126 p53.free.fr/p53.iarc.fr/
Nearly all mutant P53s at hotspot residues have lost or retain only a very weak transactivation function	55, 82, 83
A significant fraction of tumor-associated mutant P53s, that are found at moderate to low frequency in cancer, retains some level of transactivation potential	126, 51, 61
Transactivation-based classification of mutant P53s appears more important for genotype-phenotype correlations than dominant-negative effects	83
The yeast functional assay was used to determine highly specific mutation fingerprints in the human P53 cDNA sequence	28, 39
ΔN-P63α showed higher activity than TA-P63α towards REs containing a higher frequency of non-consensus bases and a reduced frequency of the CATG sequence at the CWWG core motif ΔN- and TA P63β isoforms as well as P73α and P73β isoforms or ΔN or ΔC-P53 proteins do not exhibit differences in relative transactivation specificity	81
Most of P63 missense mutations corresponding to P53 hotspot residues were transcriptionally inactive	56
P63 at 37°C is able to activate p21/WAF1, BAX, MDM2 promoters while P53 transactivates p21/WAF1, BAX, MDM2 and 14-3-3σ targets. At 30°C P63 lose activity also on p21/WAF1, while P53 shows no activity on BAX and 14-3-3σ targets).	116
P63 germline mutations associated with Ectodermal Dysplasias can be functionally heterogeneous	84
P53 mutant alleles show differences in their potential to inhibit wild type P73 transactivation function	79
Mutations at conserved amino-acids of loops L1 and L3 in the DNA binding domain (DBD) of P53 family proteins tune the transactivation potential nearly equally for P53, P63, P73	15
p65/RELA and a transcription competent p50/NFKB1 exhibit distinct transactivation specificities	113
The inhibitory function of IκB appeared to be influenced by the RE type and by the specific NF-κB homodimer being tested	113
A growth assay was used to evaluate the potential of CP-31398 molecule to stabilize the P53 protein	134
The possibility to use yeast to test small molecules targeting was established and validated using the known MDM2 or MDM4 P53/P73 inhibitors, Nutlin-3A and SJ-172550, as well as using RITA, a small molecule that can reactivate wild-type P53 in cancer cells	4, 31, 64, 124, 125
The NF-κB inhibitors BAY 11-7082 and ethyl pyruvate showed an inhibitory effect that was distinct in the case of expression of p65 alone or of p50/p65 co-expression, and also in relation to the RE tested	113

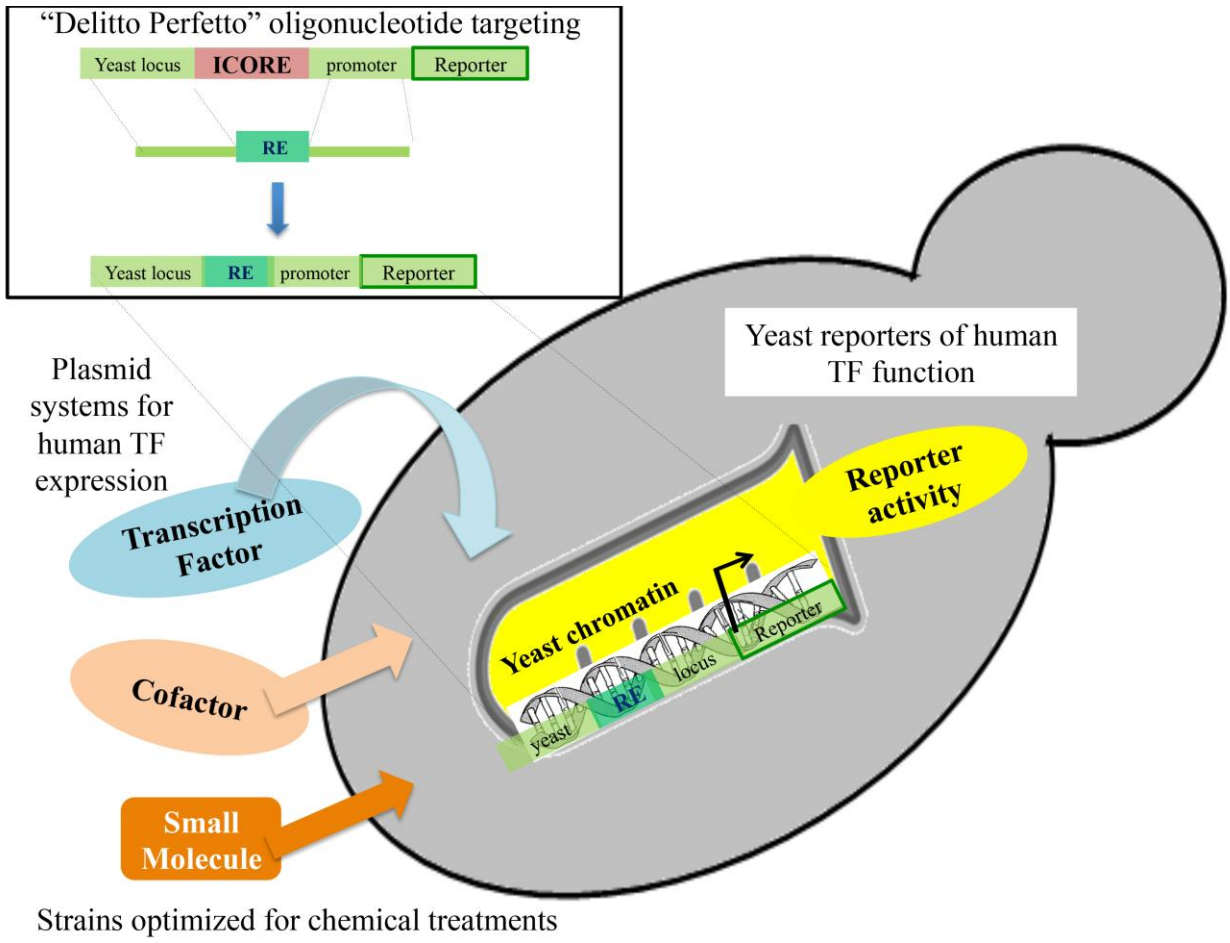

Figure 1: **Establishing human TF reporter strains by promoter targeting at chromosomal loci using *Delitto Perfetto*.**

Schematic description of the *Delitto Perfetto* to rapidly target desired TF REs upstream of a minimal promoter controlling the expression of a reporter gene [46, 142]. The experimental protocol is described in the main text. The recent construction of sgRNAs and Cas9 expression systems in yeast [22], can further simplify targeting of desired yeast promoter and enable the construction of multiple reporter strains. Human transcription factors and cofactors can be then expressed in the obtained strains by transformation of selectable plasmids, exploiting one of various promoter systems (Supplementary Table 1) and low-copy or multi-copy selectable plasmids. Transformants can then be processed for the functional assay, including the option of treating cell cultures with small molecule acting as agonist or antagonist of TFs. Yeast strains with improved chemical uptake have been developed [128]. The functional assay can be performed in small volume format. Considering the ease of constructing isogenic reporter strains

that differ only for a specific TF RE and the possibility to regulate the expression levels of TF proteins, yeast can be considered as a sort of test-tube to quantify i) the impact of TF mutations and/or RE sequence variants, ii) the co-expression of cofactors, and iii) the efficacy of small molecules.

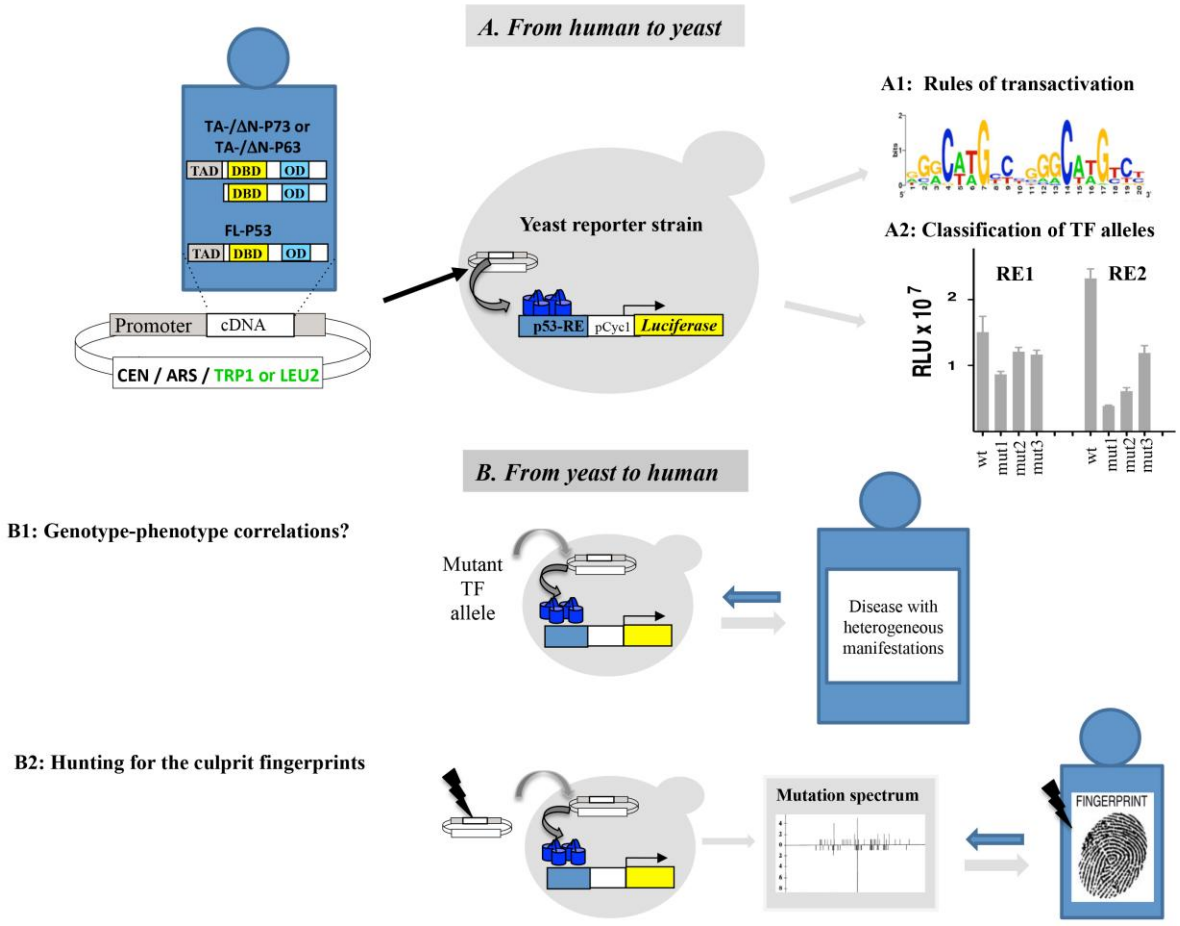

Figure 2. From humans to yeast, and back: the example of the P53 family.

The range of applications of the yeast functional assay is summarized considering as an example the studies with the P53 family of TFs –see text for details and references-. From human to yeast. **A1**. The functional assay with wild-type TFs in yeast allow to determine molecular rules that govern transactivation specificity depending on the type of TF (different isoforms), the amount of TF (constitutive expression from a *pADH1* promoter *versus* inducible expression from *pGAL1* promoter) and the sequence of the RE. The final output of such extensive analysis, using the WebLogo tool for a graphical summary, highlights the features of the RE in terms of over-representation of specific bases at distinct positions that result essential for transactivation function. **A2**. Results of transactivation assays with mutant TFs led to the discovery of functional heterogeneity among TF alleles. This heterogeneity is evident in terms of specific mutant TFs able to activate transcription from certain REs, but not from others. From yeast to human. **B1** The classification of disease-associated mutant TFs based on relative transactivation potential in

yeast can be a tool for interpreting the heterogeneity of clinical manifestations of correlated syndromes, suggesting possible genotype-phenotype correlations. **B2** The use of P53 cDNA as mutagenesis target gene in yeast is a tool for determining specific mutational fingerprints associated with environmental mutagens or chemotherapeutics. In the context of molecular epidemiology studies, the comparison of the experimentally induced mutation spectra with those obtained in human cancer studies can trap the culprit.