

1 **Context dependent activity of p63-bound gene regulatory elements**

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10 **ABSTRACT**

13 The p53 family of transcription factors regulate numerous organismal processes including the
14 development of skin and limbs, ciliogenesis, and preservation of genetic integrity and tumor
15 suppression. p53 family members control these processes and gene expression networks
16 through engagement with DNA sequences within gene regulatory elements. Whereas p53
17 binding to its cognate recognition sequence is strongly associated with transcriptional activation,
18 p63 can mediate both activation and repression. How the DNA sequence of p63-bound gene
19 regulatory elements is linked to these varied activities is not yet understood. Here, we use
20 massively parallel reporter assays (MPRA) in a range of cellular and genetic contexts to
21 investigate the influence of DNA sequence on p63-mediated transcription. Most regulatory
22 elements with a p63 response element motif (p63RE) activate transcription, with those sites
23 bound by p63 more frequently or adhering closer to canonical p53 family response element
24 sequences driving higher transcriptional output. The most active regulatory elements are those
25 also capable of binding p53. Elements uniquely bound by p63 have varied activity, with p63RE-
26 mediated repression associated with lower overall GC content in flanking sequences.

27 Comparison of activity across cell lines suggests differential activity of elements may be
28 regulated by a combination of p63 abundance or context-specific cofactors. Finally, changes in
29 p63 isoform expression dramatically alters regulatory element activity, primarily shifting inactive
30 elements towards a strong p63-dependent activity. Our analysis of p63-bound gene regulatory
31 elements provides new insight into how sequence, cellular context, and other transcription
32 factors influence p63-dependent transcription. These studies provide a framework for
33 understanding how p63 genomic binding locally regulates transcription. Additionally, these
34 results can be extended to investigate the influence of sequence content, genomic context,
35 chromatin structure on the interplay between p63 isoforms and p53 family paralogs.

36 **INTRODUCTION**

39 Transcription factors regulate gene expression networks during development and are
40 responsible for the maintenance of cellular and organismal homeostasis. These activities
41 require transcription factor interactions with DNA, usually via conserved sequence motifs within
42 cis-regulatory elements (CRE) like promoters and enhancers (Slattery et al., 2014). Sequence
43 specific transcription factor binding to regulatory elements can affect gene expression in
44 multiple, context-dependent ways, including direct recruitment of cofactors or RNA polymerase

45 and through control of local and long-distance chromatin structure. TF control of gene
46 expression is not a binary “on/off” state, and represents a range of dynamic interactions with
47 DNA dictated by sequence, chromatin, and other locally-bound transcription factors (Hager et
48 al., 2009; Ricci-Tam et al., 2021). Ultimately, understanding how DNA sequence and chromatin
49 context at CREs controls TF binding is critical for dissecting complex gene regulatory networks
50 during development and in disease.

51

52 The tight relationship between sequence-specific transcription factor activity, CREs, and gene
53 expression is especially important for lineage specification during development (Spitz and
54 Furlong, 2012; Long et al., 2016; Barral and Zaret, 2023). The transcription factor p63, a
55 member of the well-known p53 family, is a key regulator of epithelial lineage specification and
56 self-renewal (Senoo et al., 2007; Melino et al., 2015; Li et al., 2023). Extensive work using p63
57 loss-of-function mouse models demonstrates the essentiality of p63 for development of limbs,
58 digits, and craniofacial structures (Yang et al., 1998; Mills et al., 1999). These phenotypes are
59 consistent with those in humans, where p63 mutations cause multiple disorders rooted in
60 epithelial cell dysfunction, including EEC (Ectrodactyly, Ectodermal Dysplasia and Cleft lip or
61 Cleft lip and palate), Limb-Mammary Syndrome (LMS), Rapp-Hodgkin Syndrome (RMS), and
62 ADULT syndrome (Celli et al., 1999; Amiel et al., 2001; McGrath et al., 2001, 2001; van
63 Bokhoven et al., 2001; Bougeard et al., 2003). These epithelial-associated activities underlie the
64 importance of p63 in multiple organ systems during development and in post-development
65 contexts (Fletcher et al., 2011; Yallowitz et al., 2014; Richardson et al., 2017; Song et al., 2018).
66 Organismal-level phenotypes in mouse models and human disorders are consistent with the
67 indispensable role of p63 in the formation and maintenance of both the epidermis and epithelial-
68 derived cells and tissues.

69

70 Mutations within p63-bound CREs are also directly linked to human developmental disorders,
71 suggesting p63 regulation of CREs is required for development (Rahimov et al., 2008;
72 Thomason et al., 2010; Lin-Shiao et al., 2019). While multiple human disorders are linked to
73 mutations that reduce p63 function, p63 hyperactivity and gain-of-function contribute to post-
74 developmental disorders like cancer. Overexpression of p63 drives tumorigenesis in squamous
75 cell carcinomas (Ramsey et al., 2013; Saladi et al., 2017; Abraham et al., 2018), while genetic
76 rearrangements in *TP63* lead to gain-of-function activities of p63 fusion proteins important for
77 lymphoma progression (Saladi et al., 2017; Ng et al., 2018; Moses et al., 2019; Wu et al., 2023,
78 p. 63).

79

80 Like other transcription factors, p63 activity requires direct binding to specific DNA motifs within
81 CREs (Yang et al., 2006; Perez et al., 2007; Lambert et al., 2018). The *TP63* gene encodes
82 multiple transcript and protein isoforms (Mills et al., 1999; Candi et al., 2006; Murray-Zmijewski
83 et al., 2006; Sethi et al., 2015; Marshall et al., 2021; Osterburg and Dötsch, 2022), with the two
84 most prominent being TA_p63α and ΔNp63α generated from alternative promoter usage. TA_p63α
85 is an obligate transcriptional activator, and functions in preservation of genetic integrity in germ
86 cells, adult stem cell maintenance, and late-stage keratinocyte differentiation (Candi et al., 2006;
87 Su et al., 2009; Gebel et al., 2017). On the other hand, ΔNp63α's activity is strongly context-
88 dependent, and has been shown to be both a transcriptional activator and repressor (Fisher et

89 al., 2020). ΔNp63a is a pioneer factor which licenses epithelial-specific regulatory elements
90 during development (Pattison et al., 2018; Li et al., 2019; Lin-Shiao et al., 2019; Yu et al., 2021),
91 but can also bookmark chromatin structure at already established active regions or control 3D
92 interactions to repress gene expression (Bao et al., 2015; Pattison et al., 2018). ΔNp63a can
93 also locally recruit traditional co-activators, like SMAD proteins and p300 (Krauskopf et al.,
94 2018; Katoh et al., 2019; Klein et al., 2020, p. 300; Sundqvist et al., 2020), or co-repressors, like
95 HDACs (LeBoeuf et al., 2010; Ramsey et al., 2011), to regulatory elements to variably control
96 transcription (Sethi et al., 2014). The specific temporal and spatial contexts where ΔNp63a
97 performs these various transcriptional roles, and how these differential activities are regulated,
98 remain unclear.
99

100 Gene regulatory elements contain multiple transcription factor binding sites in particular
101 orientations that “code” for specific transcriptional outcomes. The combination of local sequence
102 context and transcription factor occupancy at regulatory elements ultimately controls gene
103 expression networks and vast cell fate decisions (Kulkarni and Arnosti, 2003; Zaret and Mango,
104 2016; Halfon, 2020). We implement STARR-seq MPRA technology to address whether
105 sequence content and context of p63-bound gene regulatory elements might explain differential
106 p63 activities like transcriptional activation or repression (Arnold et al., 2013). We identified
107 sequence features within and around p63 binding sites that influence p63-specific activities.
108 p63-mediated repression is most associated with local GC content and the presence of nearby
109 motifs for known transcriptional repressors. p63-mediated activation is influenced by specific
110 classes of p63 response element (RE) DNA motifs that also permit binding by p53. p63-bound
111 CRE activity changes across different epithelial cell contexts with this variation potentially
112 regulated by changes in p63 expression and flanking transcription factor motifs. ΔNp63a
113 occupancy is only weakly correlated to transcriptional output unlike strong transactivators like
114 p53. However in the context of expression of a different isoform of p63, TAp63β, transcriptional
115 activation greatly increases, suggesting that isoform switching is a mechanism that controls the
116 activity of p63-bound regulatory elements.
117

118 RESULTS

119 ***Examination of the transcriptional regulatory potential of p63-bound elements***

120 To explore how ΔNp63a, hereby referred to as p63, controls epithelial gene regulatory
121 networks, we measured transcriptional activity of putative cis-regulatory elements (CRE) bound
122 by p63 using a massively parallel reporter assay (MPRA). We selected candidate CREs from a
123 recent meta-analysis examining p63 ChIP-seq binding across multiple human epithelial-derived
124 cell lines (Riege et al., 2020) and cloned them into a reporter system using the STARR-seq
125 strategy (Muerdter et al., 2018; Neumayr et al., 2022)(Fig. 1A). We selected candidate CREs
126 where p63 binding was observed in at least 8 independent ChIP-seq experiments, resulting in
127 17,310 elements. Sequences were cloned from single-stranded oligo pool where the likely p63
128 response element (p63RE) was placed in the center of the oligo, flanked by up to 52 nucleotides
129 on either side of genomic context depending on the length of the identified p63RE. To
130 understand the specific role of p63 in transcriptional regulation by the selected CREs, we
131

133 created two variants predicted to disrupt p63 binding. We performed conservative substitutions
134 of nucleotides enriched at any single position with greater than 80% frequency (p63RE mut)
135 (Fig. 1B, Table S1). We also performed a random nucleotide shuffle of the predicted p63RE
136 located at the center of the CRE as a control for disrupted p63 binding (p63RE shuffle). Finally,
137 nucleotides flanking the p63RE (p63RE flank) or the entire CRE (full shuffle) were shuffled, all
138 while preserving GC content of the original CRE sequence. The sequence of all variants can be
139 found in Table S1.

140
141 We next examined the genomic and chromatin context of these elements to better understand
142 how these characteristics might relate to their observed transcriptional output. Although many
143 p63 binding events are intragenic (Fig. 1D), less than 20% of those are within 5kb of the
144 transcriptional start site (TSS). Most sites are localized over 5kb from the TSS suggesting
145 potential function as distal regulatory elements (Fig. 1C). ENCODE candidate Cis-Regulatory
146 Element (cCRE) classification suggests that most of these p63-bound regions display distal
147 enhancer-like signatures characterized by accessible chromatin and stereotypical histone
148 modifications such as enrichment of H3K27ac and lack of H3K4me3 (Fig. 1E) (Moore et al.,
149 2020). The next largest group overlaps the cCRE designation of NA which includes both
150 heterochromatin and “quiescent” chromatin lacking known chromatin-based features of
151 regulatory DNA. On average, 80% of these p63-bound elements are found in open chromatin
152 regions of basal epithelial cell types (Fig. 1F) (Thurman et al., 2012; Sheffield et al., 2013). In
153 contrast, most p63 binding sites are found in closed chromatin regions in all other cell types. We
154 next examined the distribution of chromatin features surrounding our MPRA elements using
155 chromHMM, which defines categorical chromatin states across multiple cell lines and conditions
156 (Fig. 1G) (Ernst and Kellis, 2015; Vu and Ernst, 2022). In three p63-positive epithelial cell lines
157 including the model mammary epithelial line MCF-10A, we observe enhancer-like enrichment at
158 greater than 45% of the MPRA elements compared to an average of approximately 20% in non-
159 epithelial cell lines (Fig. 1H). The epithelial specific enhancer-like chromatin features for the
160 surveyed MPRA elements is consistent with epithelial lineage-restricted expression and pioneer
161 factor activity of p63. We chose to use the model basal mammary epithelial cell line MCF-10A
162 cell line for subsequent studies, as significant prior datasets for p63 occupancy, transcriptional
163 regulation, and chromatin context are available. In line with summary statistics from ENCODE
164 cCRE and chromHMM, the majority of our MPRA regions bound by p63 are enriched for
165 H3K27ac and H3K4me2, but depleted for H3K4me3 (Fig. 1I), suggesting these elements have
166 primarily enhancer-like qualities in MCF-10A.

167
168 We then assayed the activity of these p63-bound CRE to investigate sequence requirements for
169 p63-dependent transcription in MCF-10A. Under basal conditions, these cells primarily express
170 the p63 isoform ΔNp63α, which is a context-dependent transcriptional activator or repressor
171 (Fisher et al., 2020). We performed two biological replicates by transfecting the STARR-seq
172 p63RE library into MCF-10A, isolated total RNA, and then specifically amplified and deep
173 sequenced the self-transcribed CRE. Plasmid DNA pools were also sequenced as a
174 transfection control, and CRE-driven RNA expression was quantified as a ratio of RNA:DNA.
175 While 17,310 candidate p63-bound CREs were originally selected for analysis, synthesis,
176 cloning, and experimental dropout reduced the number of regions used in downstream

177 experiments and analysis to 13,696. We only included regions where all five variants were
178 found in the DNA and RNA libraries at sufficient depth (Table S2, Materials and Methods).
179
180 First, we examined the role of the p63RE in mediating transcriptional activity. Mutation of either
181 the entire p63RE (RE shuffle) or of specific nucleotides predicted to be critical for p63 binding
182 (RE mut) substantially reduced transcriptional activation ($p \leq 1.00e-04$) (Fig. 1J). Similar
183 reductions in activity were seen when the entire CRE was shuffled, as expected by the loss of
184 all native transcription factor binding sites. On the contrary, shuffling DNA sequence flanking the
185 p63RE, and thus disrupting binding of other transcription factors, did not dramatically affect
186 CRE-mediated transcription. These data suggest p63-bound CREs are more dependent on the
187 central p63RE for transcriptional activation than other potential TF binding sites in flanking
188 genomic context, similar to previous observations of the central importance for the p53 family
189 RE at regulatory elements (Janky et al., 2014; Verfaillie et al., 2016; Sahu et al., 2022).
190

191 ***Influence of p63 and p53 occupancy on cis-regulatory element activity***

192

193 Given the importance of the central p63RE on CRE activity, we next investigated how p63
194 occupancy and enrichment at these elements influences transcriptional activity. We initially
195 selected p63-bound regions for study that were identified in a meta-analysis of p63 ChIP-seq
196 binding in between 8 and 20 independent ChIP-seq studies from different epithelial cell types,
197 but did not contain p63 binding data from MCF-10A (Riege et al., 2020). We therefore used
198 MCF-10A p63 ChIP-seq data from a prior study to examine how *in vivo* p63 enrichment was
199 linked to CRE activity (Karsli Uzunbas et al., 2019). Increasing p63 enrichment in MCF-10A
200 cells corresponds with increased p63 occupancy across epithelial cell types (Fig. 2A). In
201 general, more ubiquitous p63 occupancy across cell types relates to p63 enrichment in MCF-
202 10A, although significant variation exists across the range of binding events (Fig. 2A). CREs are
203 more active when p63 binding occupancy is more ubiquitous compared to sites where p63
204 binding is restricted or cell-line dependent (Fig. 2B). On the contrary, MPRA activity is poorly
205 correlated with p63 ChIP-seq enrichment in MCF-10A cells (Fig. 2C, Spearman $p=0.127$).
206 These observations suggest that ubiquitous p63 binding, those events observed across many
207 cell types, is more closely linked with transcriptional output than p63 binding enrichment as
208 measured by ChIP-seq.
209

210 Many p63 binding sites are shared by its family member p53, which is a near universal
211 transactivator (Fischer et al., 2014; Sahu et al., 2022). Prior p63 ChIP-seq meta-analyses
212 identified distinct p63 response element (RE) classes that differ primarily in their ability to
213 support binding of p53 (p53RE+p63RE) or p63 only (unique p63RE) (Riege et al., 2020).
214 Therefore, we examined whether intrinsic differences in p63RE motifs and overlapping binding
215 with p53 might better reflect the observed transcriptional activity. CREs containing the
216 p53RE+p63RE motif type were significantly more active than those with a unique p63RE (Fig.
217 2D), and saw a greater drop in activity when the central motif was lost. Similar to p63, p53
218 binding observations correlate with transcriptional output (Fig. 2B,E). Unlike our observations
219 with p63, p53 binding strength from MCF-10A cells is better correlated with CRE activity (Fig.
220 2F, Spearman's $\rho = 0.405$).

221
222 We next measured MPRA activity in MCF-10A *TP53*^{-/-} cells (Fig. 2G) to parse specific
223 contributions of p53 versus p63. CRE activity is significantly reduced in *TP53*^{-/-} relative to WT
224 MCF-10A cells (Fig. 2H). However, we also observe an additional significant reduction in activity
225 when the central p63RE is mutated in *TP53*^{-/-} conditions. Notably, in aggregate, p53RE+p63RE
226 elements are more active than unique p63 CREs even in the absence of p53 (Fig. 2I). These
227 data suggest that although p53 drives a substantial proportion of transcriptional activity for these
228 CREs, p63 still functions as an activator at p53RE+p63RE motifs even in p53's absence. They
229 also suggest that inherent sequence differences may contribute to differential transcriptional
230 output of p63-bound CREs.
231

232 ***Intrinsic response element sequence differences and GC content contribute to p63 and***
233 ***p53-dependent transcriptional activity***

234
235 Initial analysis suggests that p63 primarily functions as a transcriptional activator, with mutations
236 to the p63RE significantly reducing transcriptional output (Fig. 1J) despite p63 enrichment being
237 poorly correlated with transcriptional activity (Fig. 2C). CREs containing p53RE+p63RE
238 sequences are substantially more active than those containing motifs supporting only p63
239 binding (Fig. 2D) independent of whether p53 or p63 is engaged (Fig. 2I). The extent to which
240 variation in half-site sequence and other intrinsic DNA information within a p53 family response
241 element leads to differential binding kinetics and activities remains an open question in the field
242 (Szak et al., 2001; Safieh et al., 2023).

243
244 p53RE+p63RE motifs were originally subdivided into five categories based on differences in
245 occupancy and abundance in p53/p63 ChIP-seq datasets: primary, secondary, tertiary,
246 quaternary, and quinary (Fig. 3A) (Riege et al., 2020). Primary motifs are considered the
247 canonical p53 family motif, containing two canonical CWWG half-sites separated by a 6bp
248 spacer (el-Deiry et al., 1992; Castro-Mondragon et al., 2022). CREs containing these elements
249 support higher enrichment of p63 (Fig. 3B) and p53 (Fig. 3C) and are more active (Fig. 3D)
250 compared to the other classes. The activity of secondary, quaternary, and quinary elements
251 descend in that order (Fig. 3D), as do p63 and p53 occupancy (Fig. 3B,C). CRE activity
252 significantly decreases when nucleotides critical for p53/p63 binding are mutated in both primary
253 and secondary motifs (Fig. 3D). Because we selected CRE sequences based on p63
254 occupancy alone, our assays ultimately did not contain any tertiary p53RE+p63RE motifs.
255 Likely due to their limited number in this dataset, mutation of quaternary (n=50) and quinary
256 (n=82) motifs lead to a small, but not statistically significant, decrease in CRE activity (Fig. 3D).
257 Similar to p53, p63 activates both primary and secondary motif-containing elements, but without
258 a strong preference for primary motifs (Fig. 3E).
259

260 We next examined whether CRE activity might relate to specific classes of the unique p63RE
261 motifs (Fig. 3F). The primary and tertiary motifs resemble canonical p53 family motifs, with two
262 half sites separated by a 6bp spacer. Secondary, quaternary, and quinary motifs are
263 characterized by the presence of a single half-site coupled with an incomplete second half-site.
264 Senary motifs, relatively lowly represented in the test sequences, generally contain a single,

265 weak half-site. Septenary motifs were excluded from this analysis due to low representation
266 ($n=2$). The relationship between unique p63RE motif type and CRE activity is more nuanced
267 than for those with p53RE+p63RE motifs. p63 enrichment is highest at primary and quaternary
268 elements (Fig. 3G), but tertiary elements drive the highest level of CRE expression (Fig. 3H,I).
269 Loss of the central element leads to statistically significant loss in CRE activity for primary,
270 secondary, tertiary, and quaternary elements, with quinary and senary motifs driving the lowest
271 expression and having the least dependence on the central motif. These observations are
272 similar in the presence and absence of p53 consistent with lack of p53 binding at these sites
273 (Fig. 3I)

274
275 Increasing p63 ChIP-seq enrichment is not coupled to increased activity (Fig. 2C), and different
276 p63RE classes contribute to, but do not explain, differential CRE activity. We sought to
277 determine whether other local sequence features might provide additional insight into p63-
278 dependent CRE activity. Previous studies demonstrate dinucleotide repeat motifs (DRMs) are
279 an indicator of regulatory sequence activity (Yanez-Cuna et al., 2012; White et al., 2013;
280 Colbran et al., 2017). Dinucleotide content was generally similar within CRE classes with the
281 exception of increased GC and CG DRM enrichment in unique p63RE and increased AT and
282 TA enrichment for p53RE+p63RE (Fig. 3J). Increased AT and TA dinucleotide content in the
283 p53RE+p63RE likely reflects the strong preference of p53 for these dinucleotides in half-sites
284 (Fig. 3A,3J). CG dinucleotide enrichment and subsequent methylation could potentially explain
285 reduced activity of unique p63RE compared to p53RE+p63RE, but these observations require
286 further study.

287
288 Differences in the distribution of dinucleotides led us to ask whether overall GC content might
289 vary between the two p63RE motif classes, as increasing GC content is linked to increased
290 activity of regulatory elements (Colbran et al., 2017; Lecellier et al., 2018). Unique p63RE have
291 higher overall GC content and these differences are primarily in regions flanking the central
292 p63RE (Fig. 3K). CREs containing p53RE+p63RE motifs are more active despite lower overall
293 GC content relative to unique p63RE. We observe a strong trend between increasing GC
294 content and increasing CRE activity for p53RE+p63RE in the absence of p53 (Fig. 3E,M), which
295 is not observed when p53 is present (Fig. 3D,M). For unique p63 motifs, CRE activity (Fig. 3H,I)
296 closely matches trends in GC content in WT and *TP53*^{-/-} conditions (Fig. 3L), perhaps
297 suggesting increased GC content between elements can overcome the absence of a strong
298 transactivator like p53.

299
300 Taken together, these data suggest that specific classes of p63REs lead to modest differences
301 in p63-dependent CRE transcriptional output. These observations suggest that for elements
302 regulated by p53, motif type and p53 occupancy/affinity are important determinants of high
303 transactivation relative to other sequence-intrinsic features, like GC content. On the contrary,
304 these elements are p63-dependent, but increased p63 enrichment and p63RE motif features do
305 not directly result in higher transactivation.

306
307 ***Local sequence content and co-occurring transcription factor motifs are associated with***
308 ***differential p63-dependent activation and repression***

309

310 Our data suggest additional intrinsic DNA sequence characteristics like GC content contribute to
311 maximal activity of p63-bound elements beyond those that directly affect recruitment of p63.
312 p53-bound elements are more active and DNA motifs with higher occupancy lead to higher
313 overall transcriptional output. Our data suggest that loss of p63 binding via mutation of the
314 central p63RE leads to a marked decrease in CRE-driven transcriptional activity. When viewed
315 in aggregate, these results suggest p63 predominantly activates transcription. The ΔNp63a
316 isoform of p63, which is the predominant isoform in basal epithelial cells like MCF-10A,
317 mediates both transcriptional activation and repression, along with chromatin remodeling
318 activities, when binding to regulatory sequences (Bao et al., 2015; Fisher et al., 2020; Yu et al.,
319 2021). We asked whether p63 and p53 transcriptional activation might mask other context
320 dependent activities like repression by examining CRE behavior in the absence of p53. We
321 classified p63-dependent CRE activity as “activating” if mutation of the p63RE led to lower
322 activity relative to WT and “repressing” if lack of p63 binding led to more transcriptional output.
323 Overall, we observe p63-dependent transcriptional activation at nearly 30% (4044/13696) of
324 CREs and repression at only 10% (1345/13696) (Fig. 4A). Interestingly, the activity of most p63-
325 bound elements is not affected when the central p63RE is mutated, regardless of motif type
326 (Fig. 4A). These data potentially suggest that either p63 has non-transcriptional roles that
327 cannot be measured via STARR-seq-style reporter assays or that these elements are strongly
328 cell-type or context-dependent.
329

330

331 Activities varied based on the type of p63RE motif found within the CRE. Those containing a
332 p53RE+p63RE motif are almost twice as likely to require p63 for transcriptional activation (Fig.
333 4A). Primary p53RE+p63RE motifs more frequently lead to p63-dependent activation compared
334 to any other class whereas p63-mediated repression is generally similar across subclasses of
335 p63 response elements (Fig. 4B). Activation at unique p63RE sites is relatively similar across
336 motif types with the exception of those containing quaternary motifs (Fig. 4C). Because they are
337 found in only 65 total regulatory elements, we expect that quaternary motifs are unlikely to
338 broadly represent a general feature that underlies p63-dependent transcriptional activation. GC
339 content spanning the central p63RE is slightly reduced in p63-repressed elements relative to
340 those where p63 activates transcription (Fig. 4D). p63RE type and nucleotide content partially
341 reflect differences between p63-mediated activation and repression, although these effects are
342 relatively modest. Therefore, our data suggest the p63RE motif is critical, but that variation in
343 sequence content between elements is not a major determinant in p63-dependent
344 transcriptional activation and repression.

345

346 Cis-regulatory element activity is controlled by the total complement of transcription factors and
347 co-factors interacting with the element (Kulkarni and Arnosti, 2003; Jindal and Farley, 2021). We
348 asked whether sequences, and therefore other DNA binding factors, flanking the central p63RE
349 motif might contribute to activation or repression of p63-bound CREs. As expected, mutation of
350 the central p63RE led to decreased activity at p63-activated elements and an increase in activity
351 at p63-repressed elements, indicative of p63-dependent activity (Fig. 4E). Shuffling sequences
352 flanking the central p63RE led to partial loss of function for both p63-activated and repressed
elements. These data suggest that DNA sequences outside of the central p63RE contribute to

353 p63-mediated activities, but the specific mechanisms are not known. We then explored if
354 particular transcription factor motifs might be enriched in CREs where p63 activity was either
355 activating or repressing (Fig. 4F). We used p63RE-containing elements whose activity was p63-
356 independent (unchanged) as the background control to specifically identify unique motifs that
357 might contribute to either activation or repression versus general enrichment with p63RE. p53
358 family motifs are enriched in activating elements, likely reflecting the observation that most
359 activating elements contain the primary sub-motif which is most closely aligned with the
360 canonical motif models used in the HOMER motif finding algorithm (Table S3) (Heinz et al.,
361 2010; Duttke et al., 2019). Activating elements are also broadly enriched with AP-1 family motifs
362 consistent with these elements supporting transcriptional activation (Biddie et al., 2011;
363 Thurman et al., 2012; Seo et al., 2021). Elements repressed by p63 lack these canonical trans-
364 activator motifs, but are enriched for a series of known transcriptional repressors like Snail,
365 Slug, Zeb1, and Zeb2. All four of these factors have established roles in transcriptional
366 repression during epithelial-to-mesenchymal transition (Peinado et al., 2007; Kalluri and
367 Weinberg, 2009; Pastushenko and Blanpain, 2019). p63-repressed elements are also enriched
368 for motifs for a select set of lineage-specific transcription factors such as Ascl2, MyoG, Tbx5,
369 and Pitf1a, perhaps suggesting p63 and these factors might cooperate to repress key elements
370 during lineage transitions during directed differentiation (Pattison et al., 2018; Li et al., 2019).
371 Although motifs for known activators and repressors are enriched in flanking regions of p63-
372 bound CREs with specific activities, we cannot rule out that DNA shape or p63RE-adjacent
373 context might contribute to changes in p63 binding and activity as they do for p53 (Senitzki et
374 al., 2021; Safieh et al., 2023).

375

376 ***Cell identity and isoform availability influence p63-bound cis-regulatory element activity***

377

378 Our data indicate that most p63-bound CREs are not dependent on p63 for their transcriptional
379 activity in MCF-10A STARR-seq assays (Fig. 4A). p63 is a context-dependent transcriptional
380 activator and repressor whose activity is restricted to epithelial cells. While broadly important as
381 a regulator of lineage specification and self-renewal, p63 activity varies across epithelial cell
382 types. For example, p63 is amplified in many squamous cell carcinomas and is associated with
383 poor prognosis and pro-tumorigenic phenotypes (Latil et al., 2017; Saladi et al., 2017; Abraham
384 et al., 2018). We therefore asked whether p63 expression across different epithelial contexts
385 might lead to differential activity of p63-bound CREs in our assay. HaCaT are a spontaneously
386 immortalized keratinocyte line that can undergo squamous differentiation in culture and
387 preserve many of the features of normal human keratinocytes (Wilson, 2013). SCC-25 are
388 squamous cell carcinoma of the tongue, a cancer type that is highly-dependent on p63 for
389 proliferation (Thurfjell et al., 2005; Latil et al., 2017; Saladi et al., 2017; Pokorna et al., 2022).
390 Importantly, both cell lines have inactivating mutations in p53 that limit the analysis to p63-
391 dependent activities (Bamford et al., 2004). HaCaT and SCC-25 also express higher levels of
392 p63 than MCF-10A cells (Fig. 5A) (Sethi et al., 2015), allowing us to ask whether increasing
393 cellular p63 concentration might alter CRE activity.

394

395 We transfected the MPRA library into either HaCat or SCC-25 cell lines and measured
396 transcriptional output as previously described. Due to differences in transfection efficiency

397 between cell types, we ultimately recovered 8,566 elements with paired WT and mutant
398 expression data across all replicates of MCF-10A *TP53*^{-/-}, HaCaT, and SCC-25 cell lines. We
399 asked whether variation in cell type or p63 expression levels might alter the scope of p63-
400 dependent activation or repression. Overall, the percentage of p63-activated elements is similar,
401 albeit slightly lower, in HaCaT and SCC-25 compared to MCF-10A. SCC-25 cells have nearly 3-
402 fold more p63-repressed elements than the other cell lines (Fig. 5B) although we observe
403 relatively few enriched transcription factor motifs that might contribute to this repression (Table
404 S3). p63-dependent CRE activity varies across these three epithelial cell contexts as most
405 CREs have varied activity across at least two cell lines (Fig. 5C). p63 expression is elevated in
406 both HaCaT and SCC-25, although this does not appear to directly correlate with observed
407 differences in CRE activity. Nearly 30% of CREs do not require the central p63RE for
408 transcriptional activity in any condition (Fig. 5B,C), suggesting they function independent of p63.
409 These results indicate that the collective action of p63 and other transcription factors might
410 underlie the observed variability in gene regulatory element activity.

411
412 We then focused on the cell line-specific variability in p63-mediated transcriptional activation.
413 Slightly over 40% (988) of p63-activated CREs are shared between MCF-10A and SCC-25,
414 leaving substantial variability in p63-dependent activity between CREs in SCC-25 (1,287) and
415 MCF-10A (1,400). We therefore examined the differences in transcription factor motif
416 enrichment between p63-dependent CREs in MCF-10A and SCC-25. AP-1 and TEAD family
417 motifs are enriched at p63-activated CREs in both MCF-10A and SCC-25 consistent with their
418 reported roles in transcriptional activation at regulatory elements (Fig. 5D) (Biddie et al., 2011;
419 Currey et al., 2021; Seo et al., 2021). Ets, Smad, and C2H2 zinc finger-related motifs are
420 uniquely enriched in MCF-10A, while SCC-25 p63-activated CREs are enriched for multiple
421 unique transcription factor family motifs, including those from the GATA, FOX, Oct, and Maf
422 families (Fig. 5E). While additional work is needed to determine the extent to which these
423 putative transcription factors are involved, our results provide evidence that p63-dependent
424 transcriptional activity is influenced by the activity of other transcription factors in a cell type-
425 dependent fashion.

426
427 Although specific elements shift between being activated or repressed by p63 depending on
428 epithelial cell context, ultimately, p63 is still not required for activity of most p63-bound CREs
429 (Fig. 5C). p53 and p63 share highly overlapping DNA response element motifs (el-Deiry et al.,
430 1992; Perez et al., 2007; Riege et al., 2020), and p53 binding leads to near universal
431 transcriptional activation due to the presence of a strong N-terminal transactivation domain
432 (TAD) (Fischer et al., 2014; Verfaillie et al., 2016). Basal epithelial cells primarily express
433 ΔNp63α (Fig. 5A), but multiple N- and C-terminal isoforms of p63 can be expressed in different
434 cellular contexts (Marshall et al., 2021). We therefore asked whether the p63-bound CRE
435 activity might have p63 isoform-specific dependence. TAp63 isoforms are expressed in late
436 stages of keratinocyte differentiation and are required for the response to genotoxic damage in
437 germ cells (Koster et al., 2004; Truong et al., 2006; Beyer et al., 2011; Deutsch et al., 2011).
438 TAp63α drives high levels of transcriptional activation in a stimulus-dependent fashion (Deutsch
439 et al., 2011; Coutandin et al., 2016), whereas other C-terminal isoforms, like TAp63β are
440 constitutively active (Lena et al., 2021). We therefore chose to measure CRE activity in

441 response to expression of TAp63 β in order to avoid potential crosstalk with genotoxic or other
442 cell stress pathways. We transfected the MPRA library into MCF-10A cells where either a
443 control protein (GUS) or TAp63 β was expressed under doxycycline-inducible control (Fig. 5F).
444 The distribution of p63-bound CREs in control conditions that are p63-activated, repressed, or
445 independent is highly similar to our previous assays in MCF-10A and MCF-10A *TP53* $^{-/-}$ cell lines
446 (Fig. 5H vs. Fig. 4A). TAp63 β expression led to increased overall CRE activity and importantly,
447 this activity is dependent on the central p63RE motif (Fig. 5G). Nearly 70% of CREs display
448 p63RE-dependent transcriptional activation when TAp63 β is expressed, more than a 2-fold
449 increase compared to control conditions (Fig. 5H). This is most observable at CREs with unique
450 p63RE which see a 3 fold shift towards p63-dependent transcriptional activation and a near-
451 complete loss of repression in the presence of TAp63 β . Taken together, these results suggest
452 that context-dependent p63 isoform expression alters the activity of cis-regulatory elements and
453 that TA-isoforms primarily activate transcription.
454

455 Discussion

456
457 The importance of p63 in regulating epithelial cell identity is supported by extensive genetic and
458 biochemical evidence. p63 regulates epidermal development through its transcription factor
459 activity and control of epithelial-specific transcription and chromatin structure. These activities
460 require p63 binding and context-specific transcriptional regulation, but how DNA sequence at
461 regulatory elements affects p63 activity is an open question. Here, we examine whether the
462 sequence content and context of p63 binding sites controls p63-dependent transcriptional
463 activity using massively parallel reporter assays. We find that sequence content of p63
464 response element motifs influences p63 binding and transcriptional activity, but that this
465 relationship is complicated. The complex relationship between sequence and function is partially
466 due to p63 roles as both a context-dependent transcriptional activator and repressor.
467

468 Δ Np63-dependent repression is relatively rare compared to activation, as has been suggested
469 by studies combining p63 binding and global transcriptome analyses (Riege et al., 2020).
470 Repression can be mediated by C-terminal recruitment of known co-repressors like histone
471 deacetylases or through antagonism of other transcription factors, like p53 (LeBoeuf et al.,
472 2010; Ramsey et al., 2011; Woodstock et al., 2021). Our data suggest that slight variation in
473 p63RE motif sequence content, like varying GC and dinucleotide content, might partially explain
474 varying activation or repression, but this is likely only a minor contributor. Motifs for known
475 repressive transcription factors like Snail, Slug, Zeb1, and Zeb2 are specifically enriched in p63-
476 repressed elements. Interestingly, these factors are key regulators of epithelial-to-mesenchymal
477 transition (EMT) (Peinado et al., 2007), a process globally suppressed by p63 (Yoh et al., 2016;
478 Latil et al., 2017). While they may antagonize each other globally, p63 and these EMT-
479 promoting factors may have cooperative roles in repression of specific genes. p63 switches
480 between repressive and activating states during development, starting by repressing non-
481 epithelial lineage genes before switching to activation during epithelial commitment (Pattison et
482 al., 2018; Santos-Pereira et al., 2019). p63 also locally represses some TFAP2C binding sites
483 important for early epidermal specification during later stages of keratinocyte maturation (Li et
484 al., 2019). Repression in these settings results from p63-dependent alteration of local chromatin

485 structure or chromatin modification by HDACs which may not be directly measured using
486 plasmid-based MPRA style assays. The full scope of p63-mediated repression, and the extent
487 of its regulation by DNA sequence alone, might not be observable in a single terminally-
488 differentiated cell line using only MPRA tools.

489
490 The relationship between sequence identity and transcriptional output for p63-bound elements
491 is also complicated by context-dependent activity of other transcription factors binding the same
492 p63RE motif. The strongest predictor of regulatory element-driven transcriptional output from
493 our results is the presence of p63RE motifs capable of binding the p63 paralog p53 (Fig. 2D).
494 These elements are highly active and dependent on the central p53/p63 response element,
495 which was recently identified as the strongest predictor of regulatory element-driven
496 transcription (Sahu et al., 2022). The relative activity was preserved in the absence of p53
497 suggesting that p63 can also drive high-level transcriptional activation (Fig. 2I)(Fig. 4A). How,
498 though, these motifs drive higher expression by p63 is still unclear. Motif identity is linked to
499 higher enrichment and transcriptional output by p53 (Fig. 3C,D), but we did not observe any
500 such relationship for p63. Sites with higher p63 enrichment are not necessarily more active, as
501 has been observed directly for p53 (Trauernicht et al., 2023). Rather, our data suggest that
502 ubiquity of p63 binding across cell types better reflects increased transcriptional activity (Fig.
503 2B). Other features, like increasing local H3K27ac (Kouwenhoven et al., 2015; Qu et al., 2018)
504 and transcription factor motifs flanking p63REs, including those for traditional transcriptional
505 activators, contribute to p63-dependent trans-activation (Yang et al., 2006; McDade et al., 2012;
506 Sethi et al., 2017). Craniofacial development requires specific and combinatorial activity of p63
507 and other transcription factors at an enhancer for *IRF6* (Rahimov et al., 2008; Fakhouri et al.,
508 2012, 2014). Our results on p63RE affinity and occupancy are consistent with a model that
509 enhancers often contain suboptimal binding sites and use motif grammar and syntax to drive
510 appropriate developmental and stimulus-dependent behaviors (Crocker et al., 2015; Farley et
511 al., 2015, 2016; Lim et al., 2024).

512
513 p53 is generally regarded as a universal activator of transcription, whereas p63 either activates
514 or represses in a context-dependent manner (Fig. 6A) (Fischer et al., 2014). Our data provide
515 insight into how sequence context, including various sub-classes of the core p63RE motif and
516 flanking transcription factor motifs, can affect these p63-dependent functions. The mechanisms
517 controlling this switch between activities, including when p63 serves as a pioneer or
518 bookmarking factor (Bao et al., 2015; Kouwenhoven et al., 2015), are not fully understood. p63
519 is also a *bona fide* pioneer transcription factor and controls accessibility at epithelial-specific
520 regulatory elements (Kouwenhoven et al., 2015; Pattison et al., 2018; Qu et al., 2018; Karsli
521 Uzunbas et al., 2019; Li et al., 2019; Lin-Shiao et al., 2019; Santos-Pereira et al., 2019; Yu et
522 al., 2021). Massively parallel reporter assays are powerful tools to study transcriptional
523 activation and repression, but their design can limit the range of transcription factor activities
524 that can be directly measured (Inoue and Ahituv, 2015; Trauernicht et al., 2020). Most elements
525 display p63 expression-dependent chromatin accessibility in epithelial cell types (Fig. 1F) but do
526 not rely on ΔNp63a for their observed transcriptional activity (Fig. 4A). Roles for p63 in
527 enhancer:promoter interactions, such as those observed during p63-dependent directed
528 keratinocyte differentiation (Pattison et al., 2018; Li et al., 2019; Qu et al., 2019), would be

529 difficult to measure in a non-genomic context. One other possibility to be investigated in future
530 studies is that many elements require p63 for *in vivo* chromatin accessibility but not for direct
531 transcriptional activation or repression. Complementary approaches, like genome-scale MPRA
532 and loci-specific genetic dissection, are likely required to fully unravel the range of p63-
533 dependent activities at regulatory elements.
534

535 The seeming lack of p63-dependent transcriptional control at a substantial number of p63-
536 bound regulatory elements led us to ask whether cell context might drive differential p63
537 activities. Enhancers are well-known to exhibit cell type and context-dependent activities
538 controlled by variable expression of transcription factors and co-factors (Spitz and Furlong,
539 2012; Heinz et al., 2015). p63 expression is strongly lineage restricted during development and
540 homeostasis and varied p63 levels have been linked to human cancers (Massion et al., 2003;
541 Graziano and De Laurenzi, 2011; Tucci et al., 2012; Pickering et al., 2014; Saladi et al., 2017).
542 Elevated p63 expression is strongly linked to pro-survival pathways in squamous cell
543 carcinomas (SCC) (Thurfjell et al., 2005; Ramsey et al., 2013; Abraham et al., 2018). These
544 collective observations led us to investigate whether p63-dependent regulatory element
545 behavior varied across epithelial cell contexts. SCC-25, a head and neck squamous cell
546 carcinoma cell line, in particular showed varied activity of p63-dependent regulatory elements
547 (Fig. 5B,C). Although more elements displayed p63-dependent repression than in MCF-10A,
548 these elements lacked specific enrichment for transcription factor motifs that might cooperate
549 with p63 to reduce transcriptional output (Table S3). In contrast, p63-dependent activation in
550 SCC-25 was coupled with enrichment of different TF motifs than those associated with
551 activation in MCF10A including a range of factors with known epithelial functions, like the Maf
552 and Forkhead families (Fig. 5E) (Lopez-Pajares et al., 2015; Napoli et al., 2024). Regulatory
553 elements with cell-specific activities appear to utilize different combinations of co-enriching
554 motifs alongside p63 (Donohue et al., 2022). The extent to which p63 amplification or other
555 transcription factor availability drives differential p63-dependent activities at gene regulatory
556 elements, both during development and disease, requires more investigation to unravel. This
557 might include combining advances in genome-scale reporter assays, single-cell spatial
558 transcriptomics, and machine-learning assisted design that have led to the ability to design
559 synthetic enhancers with defined cell type-specific activities (Taskiran et al., 2024).
560

561 Transcription factors are commonly spliced to produce various isoforms which often display
562 differential activities (Wang et al., 2008; Lambourne et al., 2024). The role of p63 at regulatory
563 elements is further complicated by the complexities of transcription factor isoforms and
564 paralogs. The constitutively active TA_p63 β isoform activated transcription at most regulatory
565 elements (Fig. 5H) similar to near-universal activation by p53 (Verfaillie et al., 2016; Peng et al.,
566 2020; Sahu et al., 2022). TA_p63 α is critical in the germline and in late keratinocyte differentiation
567 and TA_p63 α has stimulus-dependent activity unlike Δ N_p63 isoforms (Koster et al., 2004; Truong
568 et al., 2006; Beyer et al., 2011; Deutsch et al., 2011). Gene regulatory elements activated
569 uniquely by TA_p63 isoforms are bound by both TA and Δ N_p63 isoforms. The role of Δ N
570 isoforms at these elements then may be to establish and bookmark local chromatin structure for
571 later TA isoform activity, as Δ N_p63 can do for regulated cell lineage-specific p53 activity (Karsli
572 Uzunbas et al., 2019). The interplay between transcription factor paralogs with overlapping

573 DNA binding activity can drive variable gene regulatory element activity (Fig. 6B). p53 and p73
574 share considerable tissue expression and binding site overlap with p63 (Marshall et al., 2021),
575 so the extent to which paralog expression influences p63-dependent behaviors should not be
576 overlooked. Similarly, p73 may also influence p63 activity through formation of mixed p63:p73
577 heterotetramers suggesting yet another mechanism influencing regulatory element behavior
578 (Strubel et al., 2023). Our results suggest that significant additional effort should be placed into
579 identifying how cell type, developmental stage, or stimulus-dependent conditions might lead to
580 p63 isoform switching, paralog expression, and, ultimately, varied p63-dependent gene
581 regulatory activity (Fig. 6B).

582
583 In conclusion, we present a near genome-scale analysis of p63-dependent regulatory element
584 activity. Our data are consistent with varying roles of p63 in literature and suggest that while
585 sequence content is important, other local cofactors, isoform switching, paralog expression, and
586 chromatin are critical context-dependent regulators of p63-dependent CRE activity.
587 Unraveling the full scope of p63 activities will likely require multiple complementary approaches
588 including specific assays focused on p63-dependent chromatin remodeling, native approaches
589 for examining sequence content such as genome editing, and new computational tools like AI
590 and deep learning.

591
592 **MATERIALS AND METHODS**
593

594 *Cell culture*

595 All human mammary epithelial cell lines MCF-10A TP53+/+ and TP53/- (Sigma-Millipore
596 clls1049) were cultured in 1:1 Dulbecco's Modified Eagle's Medium: Ham's F-12 (Gibco,
597 #11330-032), supplemented with 5% Horse Serum, (Gibco, #16050-122), 20 ng/mL epidermal
598 growth factor (Peprotech, #AF-100-15), 0.5 µg/mL hydrocortisone (Sigma, #H-0888), 100 ng/mL
599 cholera toxin (Sigma, #C-8052), 10 µg/mL insulin (Sigma, #I-1882), and 1% penicillin-
600 streptomycin (Gibco, #15240-062). MCF10A TP53/- cells were obtained from Sigma-Millipore
601 (clls1049) and were cultured Human HNSCC cell line SCC-25 (kind gift of C. Michael DiPersio,
602 Albany Medical College) were cultured in 1:1 Dulbecco's Modified Eagle's Medium: Ham's F-12,
603 supplemented with 10% FBS (Corning, #35-016-CV), 1% penicillin-streptomycin and 400 ng/ml
604 hydrocortisone. Human transformed keratinocyte cell line HaCat and HEK293FT cells were
605 cultured in Dulbecco's Modified Eagle's Medium 1X (Corning 10-013-CV) and supplemented
606 with 10% FBS and 1% penicillin-streptomycin. All cell lines were cultured at 37°C and 5% CO₂.

607
608 *Lentiviral Production*

609 Lentiviral particles were packaged by transfecting 600ng psPAX2, 400ng of pMD2.G,
610 and 1ug of pCW57.1 containing either TA₆₃β or β-glucuronidase (GUS) control in HEK293FT
611 cells at a density of 600,000 per well. pCW57.1 (pCW57.1 was a gift from David Root, Addgene
612 plasmid # 41393 ; <http://n2t.net/addgene:41393> ; RRID:Addgene_41393), psPAX2, and
613 pMD2.G (psPAX2 and pMD2.G were a gift from Didier Trono, Addgene plasmid # 12260 ;
614 <http://n2t.net/addgene:12260> ; RRID:Addgene_12260) were obtained from Addgene. GUS
615 control plasmid was provided as part of the LR Clonase II enzyme kit (Invitrogen 11791020).
616 Lentiviral supernatants were collected at 24 and 48 hours and concentrated via spin dialysis.

617 Viral supernatants were added to MCF-10A cells with 8ug/ml polybrene for 24 hours and then
618 replaced with fresh media. 2ug/ml puromycin was added 48 hours after infection and cells were
619 selected for 72 hours.

620

621 *Western blotting*

622 Protein was isolated using custom made RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM
623 NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1% Triton x-100)
624 supplemented with protease inhibitor (Pierce, 78442). Concentration of isolated protein was
625 measured using a microBCA kit (Pierce, 23227) and 25 μ g was loaded on a 4-12% Bis-Tris
626 protein gel (Invitrogen, NP0321BOX). Protein size was analyzed using PageRuler™ Prestained
627 Protein Ladder (Thermo 26616). Membranes were blocked in 5% non-fat milk in TBS-T.
628 Antibodies used included rabbit anti- Δ Np63 antibody (Cell Signaling E6Q3O), mouse anti-p53
629 (BD Biosciences 554293), mouse anti-TAp63 (BioLegend 938102) rabbit anti-GAPDH antibody
630 (Cell Signaling D16H11), and rabbit anti- β -Glucuronidase (Sigma Aldrich G5545).

631

632 *Massively parallel reporter assay (MPRA) design*

633 MPRA query regions were selected from a recent analysis of multiple p63 ChIP-seq
634 datasets (Riege et al., 2020). Only p63 binding events observed in 8 or more independent
635 experiments and containing p63 response element (p63RE) sequences (17,310 locations) were
636 considered for analysis due to DNA synthesis constraints. MPRA regions were centered on the
637 p63RE and were extended to a total length of 119 or 120 bp based on the length of the p63RE.
638 Genomic coordinates corresponding to each p63 MPRA region were used to extract DNA
639 sequence information from the hg38 UCSC genome assembly using bedtools. Either the entire
640 MPRA sequence (full shuffle), the p63RE (shuffle), or the regions flanking the p63RE (flank
641 shuffle) were randomly scrambled, while preserving GC content, to produce three variants.
642 Position weight matrices for the p63RE were generated using consensusMatrix (Biostrings R
643 package) and visualized using seqLogo. A fourth variant (mutant) was designed where
644 consensus nucleotides found within the p63RE at a frequency greater than 75% were
645 substituted to preserve GC content. A schematic of all substitutions can be found in Figure 1A.
646 Adapter sequences were then added to the 5' (5'-TCCCTACACGACGCTCTCCGATCT) and 3'
647 (5'-AGATCGGAAGAGCACACGTCTAAC) end of each MPRA sequence. Sequences for all
648 MPRA regions can be found in Table S1. An oligo pool containing all 86,550 reporter sequences
649 was synthesized by GenScript (Piscataway, NJ, USA).

650

651 *Cloning oligo pool library*

652 MPRA oligo plasmid pool was cloned as described with the following adjustments
653 (Neumayr et al., 2022). Plasmid backbone pGB118, with added Illumina i5/i7 sequences
654 flanking the cloning site, was digested with *AgeI* and *Sall* as described (Baniulyte et al., 2023).
655 pGB118 was based on hSTARR-seq_ORI vector, which was a gift from Alexander Stark
656 (Addgene plasmid # 99296 ; <http://n2t.net/addgene:99296> ; RRID:Addgene_99296). The oligo
657 pool was amplified using Q5 polymerase and SL1947 (5'-TCCCTACACGACGCTCTC) and
658 SL1948 (5'-GTTCAAGACGTGTGCTCTC) primers for 15 cycles. Amplicons were then cloned
659 into pGB118 using HiFi assembly (NEB M0492S) and HiFi reactions were transformed into

660 DH5alpha cells (NEB C2987H) and grown in LB culture. Plasmid library was purified using
661 ZymoPURE Gigaprep kit (Zymo D4204).

662

663 *Plasmid pool transfection, library preparation and sequencing*

664 Two biological replicates were performed for each cell type and condition at
665 approximately 50 million cells per replicate and one biological replicate was performed for
666 isoform (GUS, TA_p63 β) overexpression assay. 10 μ g of plasmid library were transfected per 5
667 million cells via lipofection (Polyplus #101000046, #101000025). For TA_p63 β inducible cell line
668 and negative control (GUS) cell line, doxycycline was added at 500 ng/ml at the same time as
669 transfection. Cells were harvested after 24 hours and total RNA was extracted (Quick RNA,
670 Zymo, #R1055). 30 μ g per replicate of poly-adenylated mRNA was isolated using oligo d(T)
671 beads (NEB E7490L). Resulting mRNA was split into 6 separate reactions and cDNA was
672 synthesized using a gene-specific primer (5'-CTCATCAATGTATCTTATCATGTCTG-3') and
673 MultiScribe Reverse Transcriptase (Invitrogen, 4311235). Following cDNA synthesis, all cDNA
674 samples were pooled and one Junction-PCR reaction was performed with 16 cycles (5'-
675 TCGTGAGGCACTGGGCAGGTGTC, CTTATCATGTCTGCTCGAAGC-3') and i5 and i7 primers
676 from NEBNext Oligo Kit (E7600S) were used for Illumina barcoding with between 5 and 9
677 cycles. MPRA plasmid DNA pools were amplified with i5 and i7 primers as a control for oligo
678 representation. All libraries were pooled and sequenced as single-end, 100bp on an Illumina
679 NextSeq 2000 instrument at the University at Albany Center for Functional Genomics.
680

681

682 *MPRA library data analysis*

683 FASTQ files for plasmid DNA and enhancer RNA libraries were mapped using exact
684 pattern match using custom Python scripts. Enhancers that had less than 5 reads were
685 removed. Raw read count table is available under Gene Expression Omnibus accession
686 number GSE266670. Additionally, for Figures 1-4 were MCF-10A and MCF-10A *TP53*-/- cell
687 lines were used, only enhancers that had a match for every enhancer variant (WT, mut, shuffle,
688 flankShuffle, fullShuffle) were kept (n = 13,696). Where MCF-10A *TP53*-/-, HaCaT and SCC-25
689 (Fig. 5 B-E) or MCF-10A TA_p63 β and GUS overexpression cell lines were considered,
690 enhancers that only had a WT and mut matched variants were kept (N = 8,566 and N = 16,143,
691 respectively). All reads were normalized to the total number of reads per sample and expression
692 values are represented as RNA/DNA ratio and averaged between the replicates. Normalized
693 expression values and enhancers considered for each figure are listed in Table S2. Statistical
694 tests were performed using Python packages (SciPy, scikit-posthocs).

695

696 *Data Integration*

697 MCF-10A p63, p53, H3K27ac, H3K4me2, and H3K4me3 datasets were downloaded
698 from Gene Expression Omnibus accession GSE111009 (Karsli Uzunbas et al., 2019). Raw data
699 were mapped to the GRCh38 reference assembly using hisat2 and biological replicates were
700 combined using the merge function of samtools (Li et al., 2009; Kim et al., 2019). Enrichment at
701 MPRA regions was quantified and visualized using deepTools (Ramírez et al., 2016). p53 and
702 p63 enrichment data were quantified within 1bp bins across the entire p53/p63RE motif location
703 and heatmaps were generated using 10bp bins across a region spanning -/+ 1,000 bp from the
p53/p63RE motif. Datasets used to integrate ENCODE ChromHMM, candidate Cis Regulatory

704 Elements (cCRE), and DNase Hypersensitivity Clusters (DHS) with MPRA genomic locations
705 were obtained from repositories as specified in Table S4. Intersections between p63 CRE
706 regions and various datasets were performed using either bedTools or BigBedtoBed (Kent et
707 al., 2010; Quinlan and Hall, 2010). Motif enrichment analyses were performed using HOMER
708 (Heinz et al., 2010; Duttke et al., 2019). GC and dinucleotide content analyses were performed
709 using custom nucleotide counting scripts in Python.

710

711 *Data availability*

712 MPRA datasets from this manuscript are available under Gene Expression Omnibus
713 (GEO) accession GSE266670.

714

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716

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721 **Figure Legends**

722
723 **Figure 1.** (A) STARR-Seq MPRA design. A p63 response element (RE) binding motif was
724 centered within each putative cis-regulatory element (CRE) with a total length of up to 120
725 nucleotides. See Material and Methods and Table S1 for more information. Each wild-type (WT)
726 element has a variant with p63RE mutation (mut or shuffle), scrambled flanking region
727 (flankShuffle) or fully scrambled variant (fullShuffle). All elements preserve total GC content. (B)
728 Position Weight Matrix (PWM) of the canonical WT p63RE or a mutant generated in this study
729 (mut). (C) Distribution of CREs used in this study based on distance to the nearest RefSeq TSS.
730 (D) Fraction of CREs found in intergenic or intragenic regions. (E) Distribution of CREs within
731 ENCODE cCRE annotated functional elements. (F) Fraction of CREs occurring within DNAse
732 Hypersensitive (DHS) clusters, denoted as “open”. (G) Predicted regulatory status of CREs
733 based on chromHMM chromatin state modeling. (I) Heatmaps representing p63, H3K27ac,
734 H3K4me2 and H3K4me3 enrichment in MCF-10A cells at CRE locations used in this study. (J)
735 Activity of CRE variants in MCF-10A cell line shown as a ratio of sequenced STARR-seq RNA
736 reads to the original DNA library. *p*-values were calculated using Kruskal-Wallis test followed by
737 Dunn’s *post-hoc* test and Bonferroni adjustment (*****p*-value < 0.0001).

738
739 **Figure 2.** Box-plot showing relationship between meta-analysis-based p63 binding observation
740 score and p63 ChIP-seq enrichment in the MCF-10A cell line (A) or WT CRE activity from the
741 STARR-seq assay (B). (C) Correlation between WT or mut CRE activity and p63 ChIP-seq
742 enrichment in MCF-10A cells (Spearman’s $p=0.127$, $p=2.26e-48$ for WT comparison and $p=-0.0$,
743 $p=0.98$ for mut). (D) WT and mut CRE activity for Unique p63RE or p53RE+p63RE motif types
744 with number of each motif type indicated on the x-axis (****: *p*-value < 0.0001, Wilcoxon signed-
745 rank test) (E) Box-plot showing relationship between meta-analysis-based p53 observation
746 score and p53 ChIP-seq enrichment in MCF-10A cell line. (F) Correlation between WT or mut
747 activity and p53 ChIP-seq enrichment in MCF-10A cells (Spearman’s $p=0.405$, $p=0.0$ for WT
748 comparison and $p=-0.094$, $p=4.4e-27$ for mut). (G) Western blot analysis of p53 and ΔNp63
749 expression in MCF-10A and MCF-10A TP53^{-/-} cells. GAPDH is used as a loading control. (H)
750 p53RE+p63RE enhancer activity in WT or TP53^{-/-} MCF-10A cell lines (****: *p*-value < 0.0001,
751 Wilcoxon signed-rank test). (I) Differences in WT CRE activity between p63RE motif types in
752 MCF-10A TP53^{-/-} cells.

753
754 **Figure 3.** Analysis of p63RE motif class and CRE activity. (A) PWM of p53RE+p63RE motif
755 classes, with stars indicating nucleotide substitutions in mut variants. p63 (B) or p53 (C) ChIP-
756 seq enrichment in MCF-10A cells within each p53RE+p63RE motif class. (D) WT or mut
757 enhancer activity in MCF-10A (D) or MCF-10A TP53^{-/-} (E) within each p53RE+p63RE motif
758 class. (F) PWM of Unique p63RE motif classes, with stars indicating nucleotide substitutions in
759 mut variants. (G) p63 ChIP-seq enrichment in MCF-10A cells within each Unique p63RE motif
760 class. WT or mut enhancer activity in MCF-10A (H) or MCF-10A TP53^{-/-} (I) based on Unique
761 p63RE motif class. (J) Dinucleotide repeat motif frequency in CREs. (K) Average GC content
762 across CRE regions separated by motif type. GC content was determined using a 10 nt sliding
763 window approach. Shaded area represents a 95% confidence interval. (L) Average CRE GC
764 content of Unique p63RE (L) or p53RE+p63RE (M) within each motif class. Statistical

765 comparisons were computed using either Mann-Whitney U test (B, C, G, L, M) or Wilcoxon
766 signed-rank test (D, E, H, I). p -values are indicated as ns: $0.05 < p$, **: $0.001 < p \leq 0.01$, ***:
767 $0.0001 < p \leq 0.001$, ****: $p \leq 0.0001$.

768

769 **Figure 4.** Functional characterization of p63-dependent CRE transcriptional activity. Functional
770 activity is defined by 1.5 fold-change (WT/mut) cutoff where “Activating” is >1.5 , “Repressing” is
771 <1.5 and the remaining are defined as “Unchanged”. (A) Distribution of enhancer function in
772 MCF-10A TP53 $^{-/-}$ cells in each motif type. Distribution of enhancer function in p53RE+p63RE
773 (B) motif classes or p63 UniqueRE (C) motif classes as defined in Fig. 3A, F. (D) Average GC
774 content across enhancer region by enhancer function. Shaded area represents a 95%
775 confidence interval. (E) WT, mut and flankShuffle enhancer activity in MCF-10A TP53 $^{-/-}$ cells by
776 function (****: p -value < 0.0001 , Wilcoxon signed-rank test). (F) Top 30 enriched motifs in
777 “Activating” or “Repressing” enhancer groups relative to “Unchanged” enhancer groups. Motif
778 enrichment was performed using HOMER (Heinz et al., 2010; Duttke et al., 2019). Dot size
779 represents the fraction of CREs containing the specified motif. Color scale indicates Bonferroni-
780 corrected p -value.

781

782 **Figure 5.** Cell type and context-dependent effect on p63-dependent CRE activity. (A) Western
783 blot analysis of p63 expression in MCF-10A TP53 $^{-/-}$, HaCaT and SCC-25 cells. GAPDH is used
784 as a loading control. (B) Distribution of p63-dependent CRE function in MCF-10A TP53 $^{-/-}$,
785 HaCat, or SCC-25 cells ($N=8,566$). (C) Sankey diagram derived from (B) depicting changes in
786 p63-dependent CRE function across three cell types. Numbers indicate CRE counts in each
787 group and cell line. (D-E) Scatter plots highlighting transcription factor motifs enriched in p63-
788 activated CREs that are shared (D) between SCC-25 and MCF-10A TP53 $^{-/-}$ cell lines or
789 uniquely enriched in each (E). Dot size represents fold-change enrichment over background.
790 Color scale indicates log-transformed p -value. (F) Western blot showing Doxycycline-inducible
791 TA $p63\beta$ or GUS control expression in MCF-10A cells. Cells were treated for 8h with either 500
792 ng/ml Dox or water as vehicle control. (G) WT and mut CRE activity ($N=16,143$) in either control
793 (GUS) or TA $p63\beta$ induced cell line (****: p -value < 0.0001 , Wilcoxon signed-rank test). (H)
794 Distribution of p63-dependent CRE function in either control or TA $p63\beta$ induced cell line.

795

796 **Figure 6.** (A) Schematic illustrating differential activity of $\Delta Np63\alpha$ at *cis*-regulatory elements.
797 While sequences can elicit either $\Delta Np63\alpha$ -dependent transcriptional activation or repression,
798 many appear $\Delta Np63\alpha$ -independent. (B) Schematic depicting how p63 paralogs and isoforms
799 binding to the same element can elicit different outcomes compared to $\Delta Np63\alpha$, including high
800 level transcriptional activation by p53 and TA $p63$. Other p63 paralogs and isoforms may have
801 different activities depending on the presence of particular protein domains, and are not
802 depicted here. $\Delta Np63\alpha$ activity at *cis*-regulatory elements also can change based on cell context
803 or on the availability of local transcription factors, although the molecular grammar of these
804 interactions and how they give rise to differential function remain unclear.

805

806 **Table S1.** Relevant CRE information and sequences used in this study.

807

- 808 **Table S2.** Normalized expression values (RNA/DNA) for cell type- and CRE variant-matched
809 enhancers used in this study.
810
811 **Table S3.** HOMER knownResults output for “Activating” or “Repressing” CREs in MCF-10A
812 *TP53*-/- and SCC-25 cell lines.
813
814 **Table S4.** Data sources.

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Figure 1

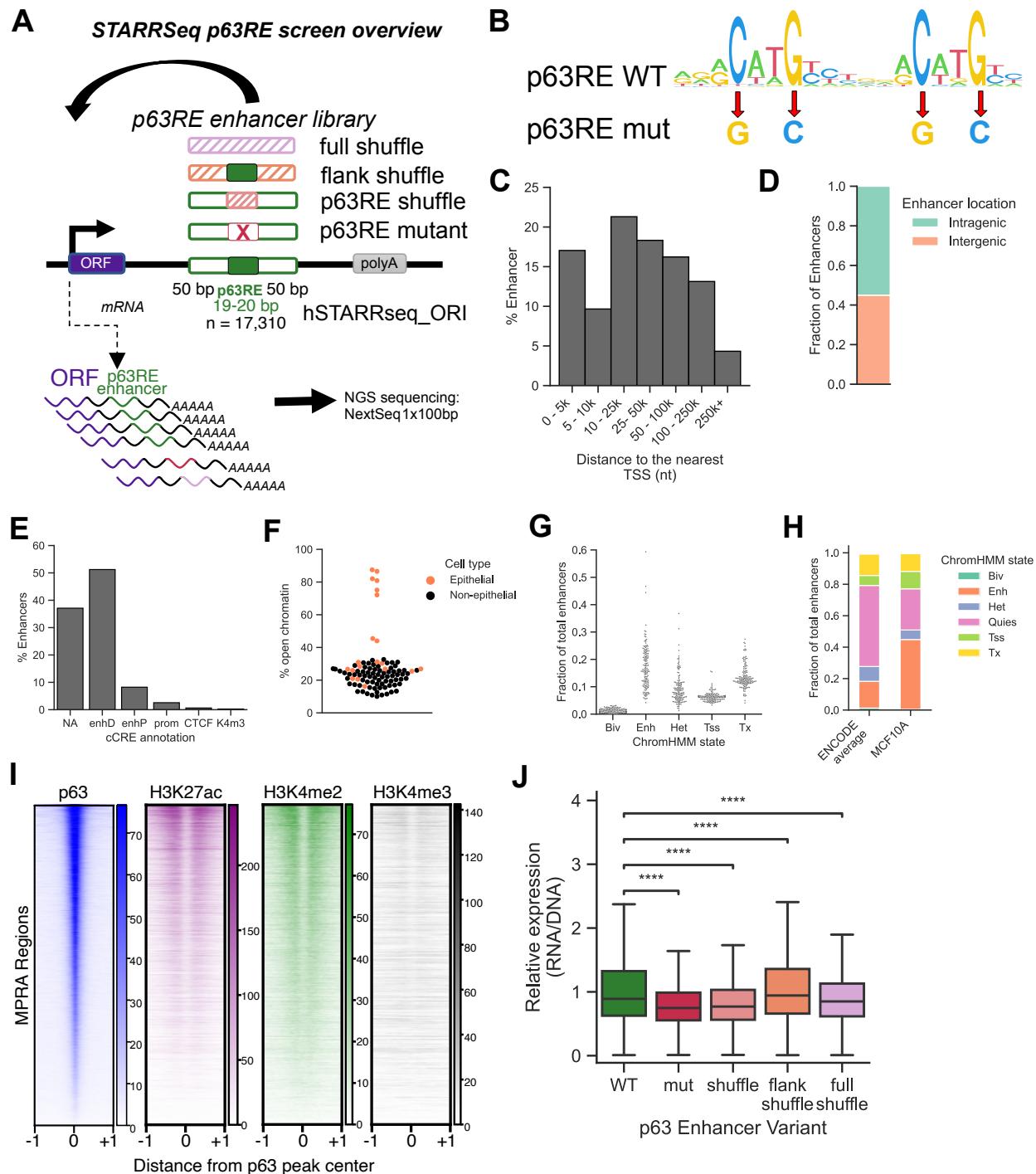


Figure 2

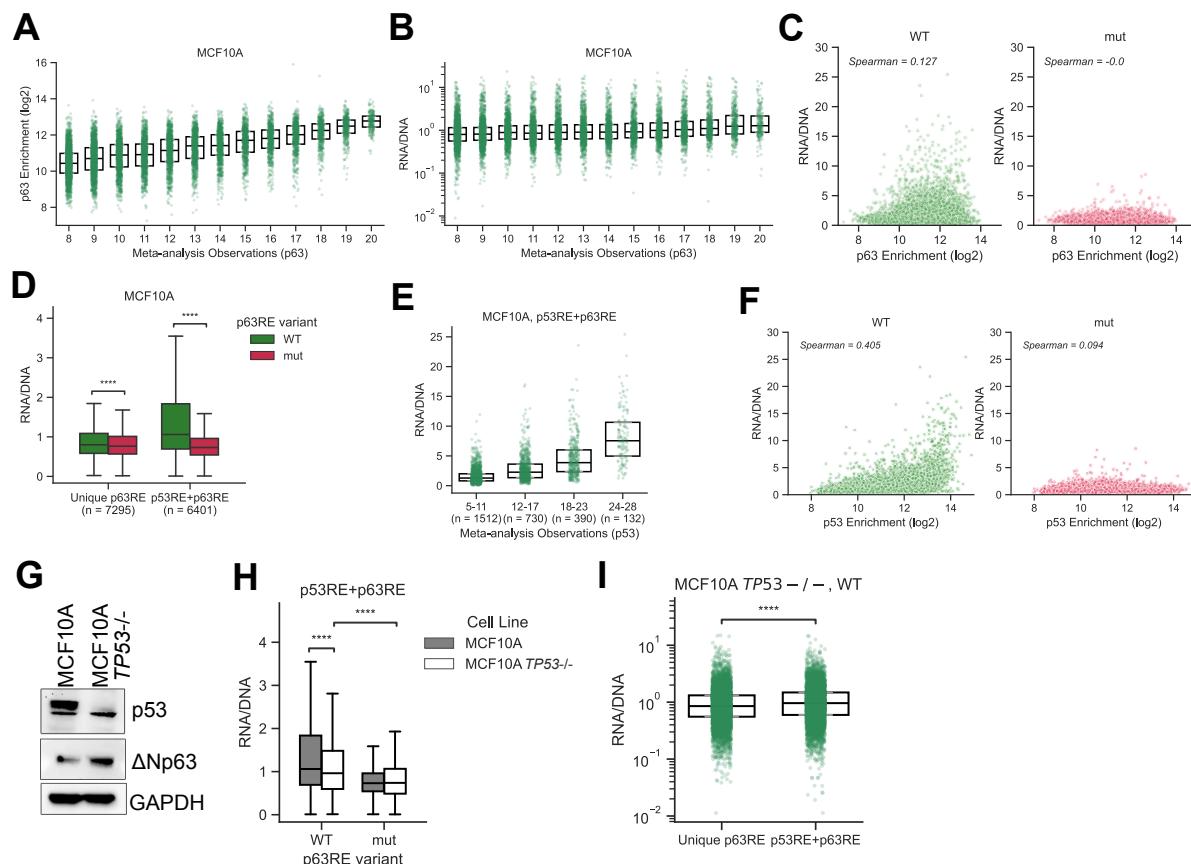


Figure 3

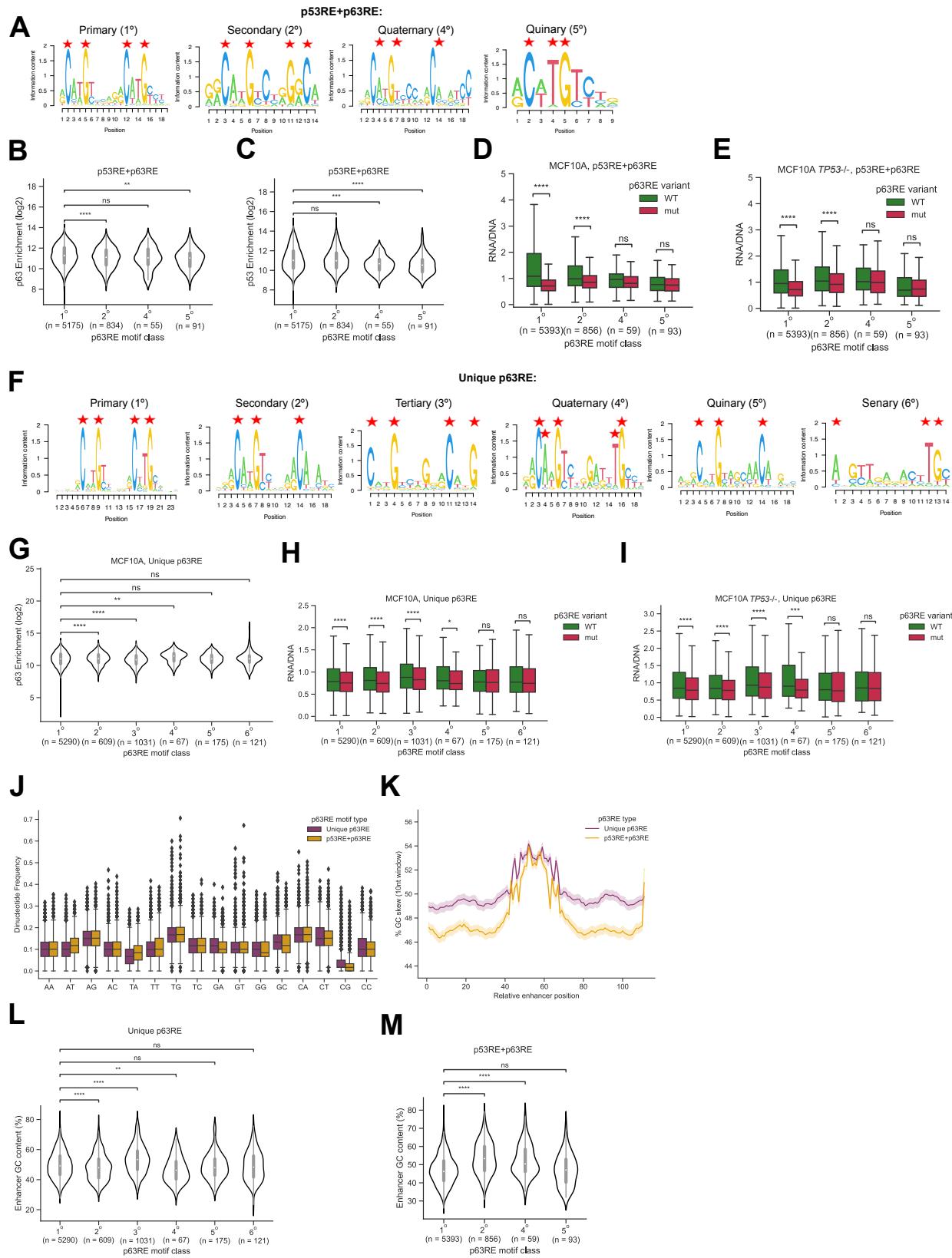


Figure 4

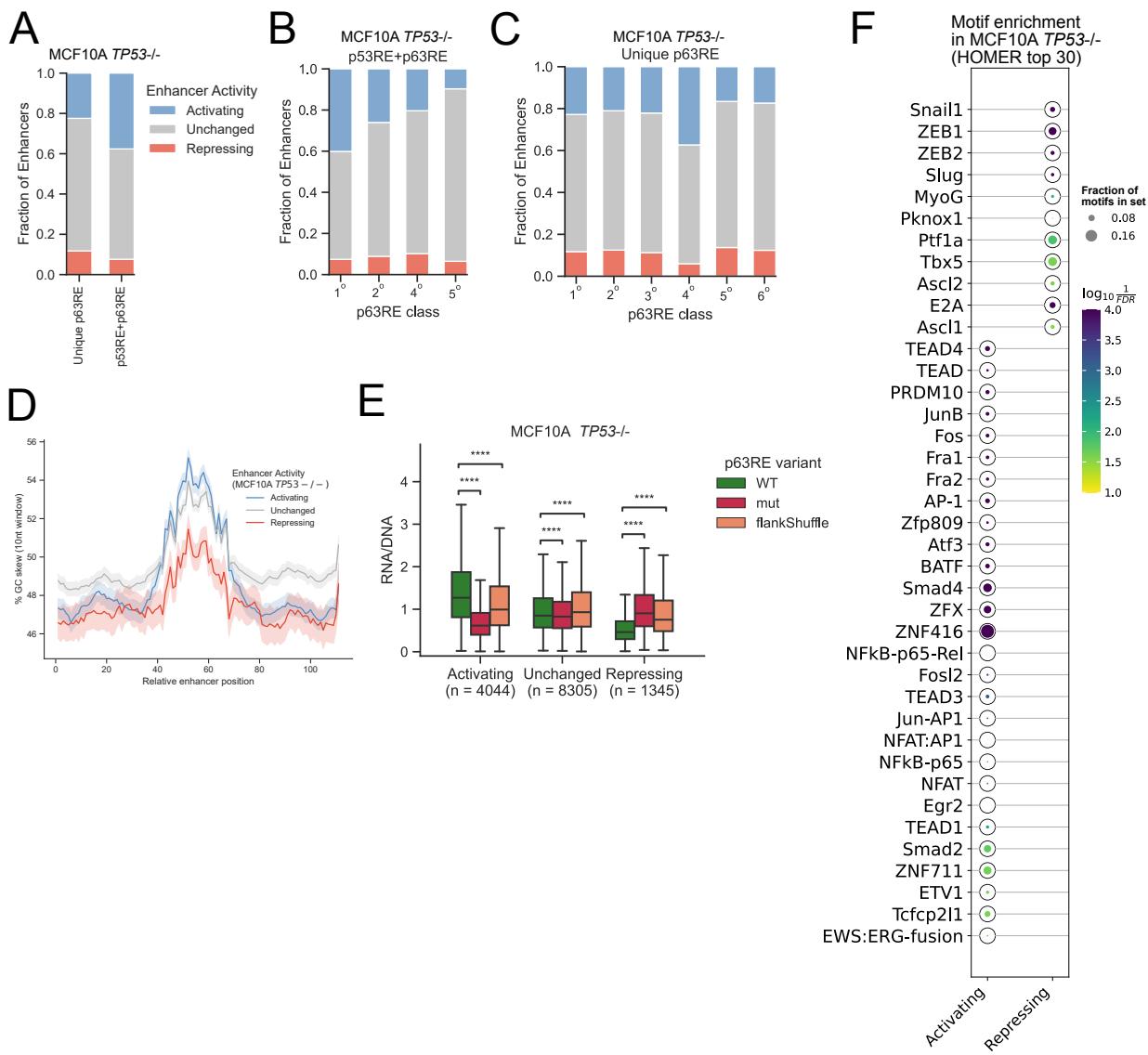


Figure 5

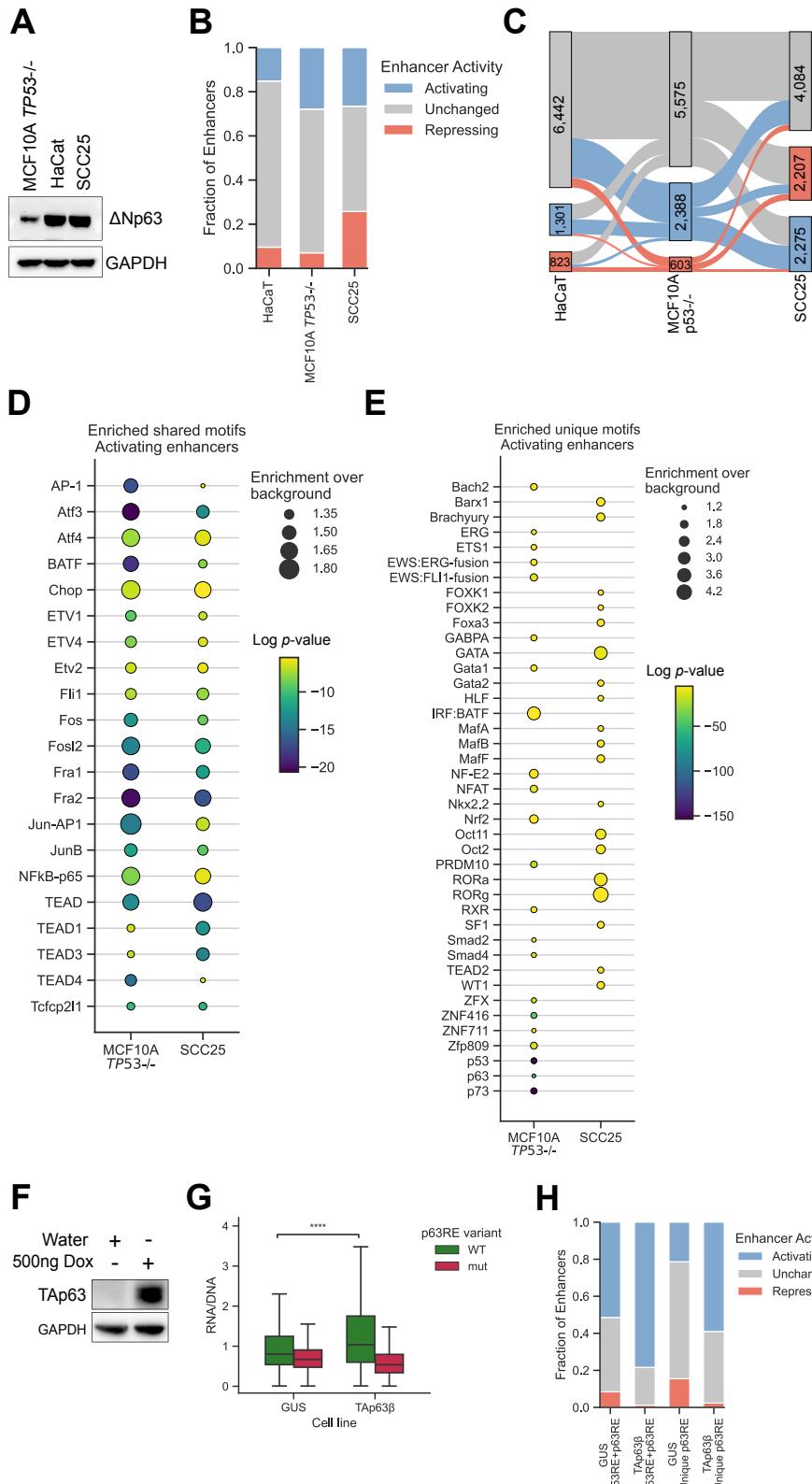
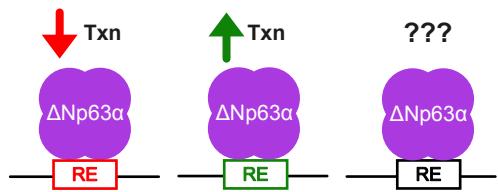


Figure 6

A



B

