Engineering elephant models of cold-adaptation and cancer resistance

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Abstract

Throughout their evolution, proboscideans have gotten larger and spread to occupy novel

environments throughout the world. These phenotypic changes have been accompanied by a suite of

genetic changes including those responsible for tumor suppression and environmental adaptation. Here

we examine a handful of woolly mammoth-specific deletions, and their potential contributions to arctic

adaptation, as well as the expanded TP53 genetic repertoire in elephants. We use CRISPR-Cas9 to

introduce mammoth-specific noncoding deletions as well as knockouts of TP53, all 29 TP53 retrogenes,

or both in combination in Asian elephant cell lines, and examine the transcriptomic response. We find

that many of the mammoth-specific deletions likely contribute to various arctic phenotypes including

vascular development, metabolism and thermogenesis, and hair and skin adaptations. Additionally, we

find that while there is considerable overlap in the DNA damage responses of the TP53 and retrogene

knockouts, there are also many unique pathways enriched in both. In particular, the retrogene

knockouts exhibit strong enrichment of many extracellular pathways suggesting they may play a role in

the tumor microenvironment and mitigating metastatic growth. This highlights the usability of cell

culture models to disentangle the effects of regulatory mutations or complex genetic systems, especially

in non-model species where the generation of animal models is not possible.

Keywords: woolly mammoths; Asian elephants; arctic adaptation; TP53; retrogenes; CRISPR-Cas9; RNA-

seq; gene regulation

1. Introduction

Gene regulation plays a significant role in the ability of species to adapt to novel environments and is believed to contribute to speciation via hybrid dysfunction [1,2]. Adaptive regulatory changes have been identified across Animalia, including in primates, mice, birds, fish, and cervids [2–5]. One of reasons for the large impact of regulatory mutations is their ability to fine-tune expression of genes during different developmental stages or tissues, in comparison to the near ubiquitous change that mutating protein sequences would have [5]. However, these tissue-specific effects, as well as the fact that regulatory sequences may be very distant from their regulatory targets, make them difficult to examine bioinformatically, and necessitate the use of cell or animal models [2].

Throughout their ~60-million-year evolutionary history, proboscidean evolution has largely followed two main trends: a general increase in size from the earliest proboscideans, *Eritherium azzouzorum* and *Phosphatherium escuillei* (~5 and 17 kg respectively); and adaptive radiation to expand into and occupy many different environments from tropical wetlands to the cold tundra [6,7] (Fig. 1A). These trends are especially apparent in the elephants (*Elephantidae*), which during the late Pleistocene included one of the largest land mammals of all time, *Palaeoloxodon antiquus* (>13 tons), and species found in various environments across Africa, Eurasia, and North America [6,7].

Woolly mammoths (*Mammuthus primigenius*) occupied dry, cold steppe-tundra environments during the coldest periods of the Pleistocene [6,8]. They evolved many physiological adaptations to survive in this environment, including a multi-layered and thicker hair coat, changes in skin and fat distribution, smaller ears and tails to minimize heat exchange, and a general increase in size and growth rate. These changes are especially striking given their closest living relative, the Asian elephant (*Elephas maximus*), is primarily found in warm topical forests and grasslands environments throughout Southeast Asia [6,8]. Previous studies have reported protein-coding changes between woolly mammoths and extant elephants which may have contributed to some of these phenotypes [9,10]. However, the evolved regulatory landscape of woolly mammoths has been largely ignored.

Peto's paradox is the observation that cancer incidence in a species does not always scale with body mass and/or lifespan [11,12]. Elephants have emerged as a key species in attempts to resolve this paradox due to their large size, long lifespan, and very low cancer risk [12,13]. Genomic analyses have identified the duplication of many tumor suppressor genes across extant elephants, including multiple retrogene copies of TP53 [12,13]. Although there is still considerable uncertainty about how or which of these TP53 retrogenes (RTGs) may confer tumor resistance, previous works have shown that the encoded protein of at least one of these retrogenes, African elephant RTG 9, induces strong cellular

responses to DNA damage [12–14]. However, most previous studies have largely focused only on African

elephant TP53 retrogenes and often in non-native genetic (e.g. plasmid expression) or cellular

backgrounds (e.g. human or mouse cells).

In this study, we examine the regulatory changes responsible for adaptation and tumor

resistance in woolly mammoths and Asian elephants in vitro. We have engineered a handful of

mammoth-specific non-coding mutations into Asian elephant cells, and observed changes implicated in

cardiac development, hair and skin function, and lipid metabolism. We further validate our cell culture

models by examining the best-studied elephant genetic pathway – their expanded TP53 repertoire. We

used CRISPR-Cas9 to disrupt TP53, all TP53 retrogenes, or both in combination in Asian elephant

fibroblasts, and examined the transcriptional profiles of these knockouts with and without DNA damage

stress, to resolve the protective roles of TP53 retrogenes (Fig. 1). Our results suggest that these cell

culture models are tremendously useful for investigating the potential effects of both adaptive

regulatory changes and tumor suppressor duplications, and generate a strong foundation of testable

hypotheses for future research.

2. Results

2.1 – Identification of mammoth deletions

Our in silico TFBS annotation predicted ~1.98 million transcription factor binding sites (TFBS)

upstream of 21,808 genes (mean 90.6/gene). We originally identified ~235k mammoth-specific

deletions, with an average size of 236 bp (min: 25; max: 13.2kb), of which 1,798 intersected our

predicted transcription factor binding sites (TFBS; Supplementary Table 3). Using previously established

methods we also predicted 629 large mammoth-specific deletions (i.e. >=500 bp; Supplementary Table

4) of which 46 were predicted to have a genome-unique guide within 25bp of their start and end

coordinates [15]. Notably we find strong concordance between both methods, with deletions identified

using our own methodology intersecting 88.1% of the large mammoth deletions identified using the

established pipeline.

2.2 – Mammoth-specific and Synthetic Regulatory Editing

We attempted to introduce one mammoth specific deletion upstream of PCNT (pericentrin), and

one synthetic deletion upstream of EIF3I (eukaryotic translation initiation factor 3 subunit I) due to its

high expression in our mesenchymal stem cell (MSC) line. PCNT mutations have implicated in the

development of smaller cranial features and olfactory development [8,16], which may contribute to arctic

adaptations present in woolly mammoths. While we were successful in generating mutations in these

regions (Fig 2A), we didn't recover differential expression of PCNT in either our pilot or verification

experiments. We did however observe differential expression of EIF3I, but only with 50% of the guide

combinations, one of which we successfully verified. Our lack of signal in PCNT and sometimes in EIF3I

underscores the effect tissue or timing has on gene regulation, and may be why we fail to observe a

signal in all cases.

2.3 – Mammoth-specific deletion differentially expressed genes

We also generated three large mammoth deletions (Deletions 3, 51, and 89) with comparable

editing efficiency in both fibroblasts and mesenchymal stem cells (Fig. 2A). Following bulk RNA-seq we

recovered a large number of differentially expressed genes (DEGs), although a significant portion of

them had very small log2 fold changes. To focus our analysis on those that were likely to be most

impacted we filtered our dataset to a minimum log2 fold change of 1 before further analysis.

Interestingly, we see a high level of variability in the DEGs affected between cell types, even given the

similarity of fibroblast and MSCs [17] (Fig. 2B), with the majority of DEGs being unique to each cell type.

This is also true even if we remove the minimum log2 fold change (Fig. S2). This suggests that these

deletions likely have cell-type specific effects and may result in very different phenotypes depending on

the cell's specific context.

2.4 – Potential impact on mammoth phenotypes

We next wanted to see if we could infer some of the functions of these deletions, particularly as

they may relate to arctic adaptations in woolly mammoths. To do this, we first screened our filtered

DEGs to identify genes within pathways of interest (Fig. 2C), and then investigated what was known

about perturbations in those genes in various cell and animal model analyses. We identified changes

that broadly fall under three phenotypes: skin and hair adaptations; vascular adaptations; and

metabolism and thermogenesis.

2.5 – Skin and Hair Adaptations

In comparison to their closest living relatives, Asian elephants, woolly mammoths have both

denser hair coverage and multiple coats (layers of hair) to protect them from the arctic cold [8]. We find

DEGs which may be related to skin and hair adaptions within each deletion, although not in every cell

type – MSC Deletion 3 (SOSTDC1 and BCL2); MSC Deletion 51 (FABP5); and Fibroblast Deletion 89

(IGFBP5). SOSTDC1 is involved in the regulation of the number, size and/or shape of hair, and is

upregulated during the anagen phase in response to depilation-induced hair regeneration [18,19]. BCL2

prevents apoptosis, and its upregulation has been shown to protect against hair cell death in the utricle

[20]. Likewise, we observe downregulation of IGFBP5 in our fibroblast Deletion 89 cells, which is a strong

negative regulator of hair development and differentiation [21]. Previous studies have also found that

overexpression of IGFBP5 produces thinner and shorter hair with aberrant morphologies [21]. The

upregulation of SOSTDC1 of these in MSC Deletion 3, and downregulation of IGFBP5 in Fibroblast

Deletion 89, suggest these deletions may contribute to the abundance and robustness of the

mammoth's coats.

We also identify FABP5 upregulated in MSC Deletion 51, which is involved in regulating water

permeability. Knockouts of FABP5 result in much lower fatty acid content in the epidermis and impaired

water-barrier function and keratinocyte migration [22]. Likewise, this gene is expressed in sebaceous

epithelial cells where it plays a role in sebaceous wax production [23]. Sebaceous wax is believed to play a

role in waterproofing and water loss, as well as thermoregulation [8,24]. Mice which have been engineered

to lack or have nonfunctional sebaceous glands suffer from abnormal hair growth/loss, skin lesions, and

compromised thermoregulation [24]. Notably, histological analyses of the few mammoth soft tissue

remains that have preserved have revealed the presence of sebaceous cells, which are almost entirely

absent in extant elephants [8,25].

2.6 – Vascular Adaptations

Vascular adaptations are also frequently observed in many arctic species. One of the best

studied adaptations within this category is an increase in heart size, likely due to the need for more

blood flow to maintain temperature and oxygen homeostasis [26,27]. Although mammoth soft tissue has

poor taphonomy, there are claims that mammoth hearts were larger than similar warm-adapted

elephants of their age and size [28]. Correspondingly, we observe genes involved in heart remodeling and

angiogenesis in four experimental conditions – MSC Deletion 3 (IGF2BP2); MSC Deletion 51 (NRG1 and

LAMA4); and Fibroblast Deletion 51 (HBEGF and PPP1R3A). Previous research has implicated IGF2BP2,

NRG1, LAMA4, HBEGF, and PPP1R3A in heart morphology and metabolism, with the overexpression of

many of these genes resulting in heart remodeling and hypertrophy [29–33]. Additionally, IGFBP2 and

PPP1R3A have also been shown to play a role in metabolic changes that accompany abnormal heart

morphologies in mice [31,32].

Accompanying these changes, we also observe differential expression in genes related to

angiogenesis – MSC Deletion 51 (LAMA4); and Fibroblast Deletion 89 (SEC14L3). SEC14L3, in conjunction

with VEGF, has a role in promoting the migration of endothelial and vein progenitor cells and

consequently angiogenesis, and its overexpression has been implicated in the proliferation and

metastasis of tumors [34]. Likewise, LAMA4 appears to be highly involved in blood vessel development.

Mouse LAMA4 knockouts appear generally phenotypically normal, but exhibit frequent hemorrhages,

and in cornea angiogenesis assays developed grossly distorted blood vessel architectures [35].

Interestingly, while both genes are strong drivers of angiogenesis, we observe downregulation of

SEC14L3 and upregulation of LAMA4 in their respective experimental conditions. Although the general

trend here is still unclear, it's possible these deletions are implicated in fine tuning angiogenesis in

mammoths.

2.7 – Metabolism and Thermogenesis

The ability to sense and respond to cold temperatures via metabolic changes is probably

amongst the most important cold adaptations. There is a suite of metabolic cascades that are triggered

in response to cold nociception, and the ability to respond to cold stimuli via thermogenesis is highly

dependent on the regulation of both glucose and lipid metabolic reserves [36]. Not surprisingly, we

recover many differentially expressed genes involved in adipocyte proliferation and lipid and glucose

metabolism in four out of our six experimental conditions.

The bulk of our differentially expressed genes are involved in adipocyte differentiation and/or

lipid metabolism – MSC Deletion 3 (IGF2BP2, PID1, and NPC1L1); MSC Deletion 51 (IGF2BP2, MSR1,

DGAT2, DUSP26, and DDIT3); Fibroblast Deletion 51 (NR4A1, EGR2, and MAFB); and Fibroblast Deletion

89 (IRF4 and IGFBP5). We observe upregulation of IGF2BP2, PID1, DDIT3, and IRF4 in their respective

experimental conditions, all of which except DDIT3 are positive regulators of adipocyte

differentiation/proliferation [37–40]. Likewise, we observe downregulation of NR4A1, EGR2, and MAFB all

of which have previously been shown to act as negative regulators of adipocyte differentiation or size [41–

43]. Unsurprisingly, almost all these genes and additionally MSR1, DGAT2, NPC1L1, DUSP26, and IGFBP5

are also involved in lipid metabolism and controlling the rates of stored versus circulating lipids [44–48]. For

example, mice induced to overexpress DGAT2 do not appear to significantly different fat pads or body

weights relative to controls but exhibit much higher levels of circulating plasma triglycerides [47]. Likewise,

MAFB deficient mice have higher body weights and amounts of body, due to large adipocytes and higher

serum cholesterol levels [43].

Accordingly, many of the above genes have been shown to be implicated in the ability to sense

and respond to cold in mouse models. GRIK2 (MSC Deletion 3) knockouts are unable to sense cold, but

not cool, temperatures and have a sever reduction in cold-specific neurons [49]. IGF2BP2 knockout mice

have better thermoregulation than controls in the short term, but have fewer and smaller adipocytes

and adipocyte precursors, and have diminished UCP1 levels, a key thermogenic protein [38]. Interestingly,

IRF4 knockouts have been shown to be more obese than wild type mice but exhibit increased cold

intolerance likely due to the cold responsive nature of IRF4 [37].

2.8 – The Asian elephant genome contains 29 TP53 retrogenes

Although our RNA-seq results suggest that the deletions we engineered are likely involved in

woolly mammoth cold adaptations, many of these phenotypes would only be measurable in organismal

models. Towards this end we have primarily referenced literature on perturbations in these genes

within mouse models, though we note that elephants are quite diverged from all common mammalian

model species [50]. To both validate our cellular models, and expand our understanding of elephant cancer

resistance, we investigated the best-studied elephant genetic system – the expanded TP53 repertoire.

We searched the annotations of the Asian elephant reference genome and identified 29

retrogene copies of TP53 (RTGs). 27 of these RTGs are found on chromosome 27, while the remaining

two are found on chromosomes 24 and 26. By comparison, the canonical copy of TP53 is located on

chromosome 19. Most of the RTGs are approximately 1.1 kb in length, roughly the same length as the

CDS of TP53 as would be expected of retrogenes, except for three (LOC126068247 – 64.1 kb;

LOC126068267 – 32.5 kb; LOC126068248 – 4.9 kb; Fig. S3). When aligned and translated in the same

frame as TP53, each RTG carries a premature stop codon, which primarily cluster in five main areas

across the homology region (Fig. 3A).

2.9 – TP53 and RTG editing and validation

We attempted to design paired Cas9 guide RNAs (sgRNAs) to produce large, disruptive deletions

in TP53 and all the retrogenes. We managed to identify two sgRNAs that specifically target TP53, which

result in the partial loss of exon 1, the entirety of exon 2, and a premature stop codon after read

through into intron 2 (Fig. 3A). We could not identify just two guides that were both predicted to have

activity across all 29 retrogenes, so we designed a third guide to ensure each RTG was targetable by at

least two sgRNAs (Fig. 3B; Fig. S4).

We electroporated fibroblast cells with Cas9 RNPs consisting of either the two TP53 guides, the

three RTG guides, all five guides together (All), or a guide predicted to have no activity in the Asian

elephant genome (Non Targ.). After approximately four days, we then collected cells, extracted DNA,

and amplified a portion of TP53 and RTG genes flanking the expected editing sites. We observed high

levels of editing across all three conditions when we examined the resulting amplicons on an agarose gel

(Fig. S5). However, due to our PCR reaction amplifying all 29 loci simultaneously, we couldn't assess

which and how many of the RTGs were being edited.

To address this issue, we sent both PCR reactions for end-to-end nanopore sequencing. We

quantified the number of observed deletions, using a custom script which marks the presence of a

deletion if 90% of the bases within a defined region were absent. We note that this is likely an

underestimate of the true editing efficiency, as this will only identify the perfect deletion product and

miss any indels and/or incomplete loss of the flanked segment of DNA. We observed high editing

efficiency in all conditions using either the TP53 guides (TP53 KO – 67%; All KO – 66%) or the RTG guides

(RTG KO – 77%; All KO – 82%), and almost no editing in the Non Targ. condition (<0.1% at both loci) (Fig.

3C). We further examined the proportion of each deletion product observed in the RTG PCRs, and noted

a clear pattern, wherein deletions between guides 2 and 3 were the most frequent, in line with

expectations about the targeting scope of those two guides (Fig. 3D).

Next, we wanted to disentangle the editing on a per-locus level for the RTG data. We mapped

the data to each unique RTG (collapsing those with identical sequences outside the editing region) and

identified the proportion of each deletion at each locus. Although we generally observe a mix of all

three deletions at each locus (some of which is likely due to cross mapping between the RTGs), we

generally observe deletions in line with our expectations based on their targeting scope (Fig. 3E). Only

two loci buck this trend, LOC126068338 and LOC126068461, both of which have a single point mutation

at position 10 within the seed region of the guide (Fig. S4). Due to the importance of this region for

guide specificity, we filtered these targets out; however, we note that at least at these two loci the

guides appear to be permissive for editing. When combined with the per-locus reads that were not

identified as containing a deletion (Fig. 3F), we observe an average of 76.8% editing efficiency across all

unique loci.

2.10 – RNA-seq analysis reveals overlapping and unique TP53 and RTG responses

Given most literature on elephant RTGs has largely focused on how these retrogenes may

interact with canonical TP53 and MDM2, we first decided to look at changes in expression of all direct

downstream targets of TP53 (Fig. 4). In the absence of stress (i.e. 10µM Mitomycin C), we see relatively

small changes in expression within most downstream targets with the exception of MDM2 and CDKN1A

in both the TP53 and All KOs. Interestingly, we note that TP53 is not identified as significantly

downregulated in the TP53 KO, suggesting maybe that nonsense-mediated decay is less pronounced in

Asian elephants. In comparison, we observe very few differentially expressed genes in the RTG KO, and

only TP53 that has a log2 fold change greater than 1. This suggests that the RTGs may play some role in

either stabilizing or increasing the abundance of p53, although likely not directly by acting as a

transcription factor as none of the RTGs have an intact DNA-binding domain (Fig. 3).

In the presence of Mitomycin C, all three conditions have similar patterns in DEGs (Fig. 4),

although typically smaller expression changes in the RTG KO. As before, we also observe that TP53 is

significantly less expressed in the RTG KO and not in the TP53 KO. Additionally, IGFBP-3, a gene which

can contribute to cell growth and apoptosis [51,52], is also downregulated in the RTG KO. We also observe

significant downregulation of SFN across all three conditions in the MMC-treated cells, in line with this

gene's known tumor suppressor function of arresting cellular replication in response to DNA damage

[53,54].

Next, we wanted to examine the different pathways enriched within each condition. Due to the

large number of genes with small changes we filtered the dataset to a minimum absolute log2 fold

change of at least 1. We then extracted the DEG lists for each condition and analyzed the overlap

between them in either the non-stressed or MMC-treated cells (Fig. 5A). We performed BioPlanet

pathway enrichment [55] on the 763 DEGs common to all three conditions treated with Mitomycin C. We

observed significant enrichment in p53 signaling pathways, as would be expected of cells exposed to a

DNA damaging agent, as well as enrichment in many DNA replication and cell cycle pathways (Fig. 5B).

We also examined the unique DEGs in the TP53 and RTG KO cells with Mitomycin C treatment

(Fig. 5C). For the TP53 KO, we see enrichment in many pathways that would be expected given the

known checkpoint and cell stress responses coordinated by TP53 (e.g. cell cycle and proliferation, DNA

repair, etc; Fig. 4D) [56–58]. By comparison, unique RTG KO DEGs are enriched in extracellular organization

and signaling/interaction pathways (Fig. 5E), with many genes that contribute to multiple extracellular

matrix related processes (Fig. 5F). This suggests that RTGs may play a unique protective role in

regulating the tumor microenvironment and limiting metastasis [59]. Additionally, the largest number of

DEGs appear to belong to the apoptotic signaling in response to DNA damage pathway, a function that

has previously been suggested for African elephant RTGs [12,14].

2.11 – Very few RTGs are likely to be expressed or functional

Given the unique function suggested by the RNA-seq of the RTG KO cells, we next wanted to see

if we could investigate which RTGs might be responsible for the changes we observed. In none of the

conditions, irrespective of treatment, did we detect differential expression of any of the RTGs.

Furthermore, we generally do not observe differences in the amount of RTGs expressed in response to

Mitomycin C treatment (Fig. S10A), with the exception of the All KO. We do observe an increase in the

amount of RTGs expressed in the RTG and All KO conditions, irrespective of Mitomycin C treatment

(significance values not shown), although this may be a lingering transcriptional effect caused by double-

strand break repair [60]. However, we do note that all but three RTGs have very low read counts

(Supplementary Table 6).

We next wanted to confirm that this pattern in RTG expression is not due to cell type or tissue

specific. We download public RNA-seq datasets for Asian elephant ovary, salivary glands, lymph nodes,

lung, thyroid, and lymphocytes, as well as examined some preliminary data generated with the same

guides in epithelial and mesenchymal stem cells, and examined RTG expression patterns in these

datasets. We observe the same general pattern, where the only three RTGs which show consistent

expression are LOC126068247, LOC126068267, and LOC126068248 (Supplementary Table 7). However,

these three RTGs are the three largest RTG genes (LOC126068247 – 64.1 kb; LOC126068267 – 32.5 kb;

LOC126068248 – 4.9 kb) based on their annotations.

To examine what was driving the expression of these RTGs, we extracted RNA-seq mapped

reads across each of these three loci and examined the frequency of reads across each locus. For two of

these RTGs, we observe that most of the reads assigned to these RTGs map thousands of base pairs

upstream of the region homologous to the TP53 CDS. In LOC126068247 and LOC126068248, we observe

almost no mapped reads within the TP53 homology region either in our data or the publicly available

datasets (Figs. S11-S14). By comparison, LOC126068267 also has peaks of high coverage upstream of the

TP53 homology region but shows similar levels of coverage across the homology region in both datasets

(Figs. S15-S16).

Given the lack of expression of most of the annotated retrogenes, we decided to compare those

that might be functional in Asian elephants with African elephant retrogene 9 (RTG 9; KF715863), on

which most previous studies have focused [12–14]. We aligned the TP53 homologous regions of all Asian

elephant RTGs, African elephant RTG 9, and the TP53 CDS, and built a maximum-likelihood phylogeny.

We observe that RTG 9 is most closely related to a clade containing two of our putatively expressed

RTGs, LOC126068267 and LOC126068248 (Fig. S10B), further suggesting that these two RTGs might be

responsible for the changes we observe. However, we do note that due to the high similarity of many of

these RTGs, the bootstrap support values within our phylogeny are quite weak.

3. Discussion

Proboscidean evolution can be characterized by a general increase in size from the 5 kg

Eritherium to the 13-ton *Palaeoloxodon*, and a dispersal from the tropical wetlands of Africa into a wide

variety of environments across the globe [6,7]. Elephants offers a unique opportunity to examine these

trends as they contain a highly evolved tumor suppressor system [12], and have closely related taxa

adapted to two distinct environments – the arctic steppe tundra (woolly mammoths), and the tropical

forests and grasslands of southeast Asia (Asian elephants) [6]. Consequently, these taxa represent key

candidates to understand both the adaptations necessary to expand into arctic environments and

maintain cellular and genomic integrity with increased body size.

Studies in other taxa have suggested that a lot of adaptive evolution takes place on the level of

gene regulation as opposed to protein function, possibly due to the reduced constraints on regulatory

motif mutations [2,5]. In this study we have investigated the regulatory role of some mammoth-specific

deletions on arctic adaptations, and the expanded TP53 repertoire found in all elephants. We do note

that all of these results should be viewed as hypotheses and testing their true impact would require the

generation of animal models to fully examine their developmental and tissue-specific effects. However,

as this is both currently not possible and ethical in elephants, we have generated many of these

mutations within elephant cells to shed some light on their possible function.

We observe many differentially expressed genes that may be involved in arctic adaptations

when we introduce our identified mammoth-specific deletions. Amongst these we see genes involved

broadly with vascular system development, skin and hair, lipid metabolism, temperature sensing, and

thermogenesis. However, we often fail to observe differential expression of genes containing or near

our perturbations, although this result is likely affected by cell type [61]. In fact, even in our larger

mammoth deletions, we observe very little overlap in DEGs between MSCs and fibroblasts. Taken

together, the results presented here are promising and provide some insight into mammoth adaptations

but likely fail to fully capture the regulatory extent of these mutations, and which likely will not become

evident until transgenic non-model species become commonplace or our ability to bioinformatically

predict causative effects within a tissue-specific context drastically increases.

We also find that only three of the twenty-nine TP53 retrogenes are expressed across an

extensive list of tissues and likely functional. Interestingly, two of the Asian elephant retrogenes most

likely to be functional are closely related to African elephant RTG9, which has been shown to induce

strong cellular responses to DNA damage [12–14] and apoptosis in mouse fibroblasts and human cancer cell

lines [12,14]. We also observe clear transcriptional differences between TP53 and RTG KO cells, including a

significant downregulation of TP53 expression in our RTG KO cells, suggesting a novel function for

elephant retrogenes. Additionally, we observe a differential enrichment of pathways when disrupting

RTGs versus TP53, indicating that while there is a high amount of overlap between these genes, they

also regulate many unique pathways. In particular, genes enriched within many extracellular

organization and signaling pathways were observed in the RTG KO population. Some of these (e.g.

extracellular matrix organization, angiogenesis) have been implicated as contributing to the

establishment of tumor microenvironments, and are gaining increased importance as vital to cancer

growth and spread [59]. This may also explain the low prevalence of malignant cancers in Asian elephants

[62].

Our work highlights the utility of cellular models to begin disentangling the complex effects of

regulatory noncoding deletions and cancer resistance genetic networks in non-model species. However,

in addition to the tissue-specific response limitations mentioned above, it is further hindered by our

reliance on primary, non-immortalized cells, although this will likely be addressed as genetic resources

for non-model species become more abundant [63,64]. Of particular importance would be stable and

readily available iPSCs, which would allow for differentiation of various tissues and more complex

organoid models to better investigate the effects of both adaptive and cancer resistance mutations,

especially where the generation of live animal models is not possible.

4. Methods

4.1 – Elephant Genomic Dataset

All bioinformatics analyses for deletion identification used previously published datasets. We

used datasets for eleven mammoths (*Mammuthus*), thirteen Asian elephants (*Elephas*), and twenty-two

African elephants (*Loxodonta*) (Supplementary Table 1). Published datasets were aligned to the *Elephas*

maximus reference genome (GCF_024166365.1) using BWA aln [65].

4.2 – Mammoth Deletion Identification

A summary of the mammoth deletion pipeline is available in Fig S1. Briefly, the *Elephas maximus*

reference genome was split into individual contigs. These were then fed into GenerateWindows.awk

(WinSize = 25; WinSlide = 10; AmbProp = 0.2), to generate windows across the genome and filter out

any with more than 20% missing data (i.e. N's). We then generated read depth files for each

mammoth/elephant alignment using samtools depth (-a to output all positions) [66], and split these by

contig. Windows were then cross referenced with depth files using another CoverageCalc.awk to extract

windows containing no mapped reads (StdevMultiplier=99), and adjacent windows concatenated using

ConcatonateWindows.awk. Windows with zero coverage identified in *Loxodonta* and *Elephas* were then

combined using bedops [67], then were used to filter the identified mammoth deletions using bedtools

intersect (-v flag to retain windows which are uniquely in the mammoth dataset) [68].

We also attempted to predict transcription factor binding sites (TFBS) upstream of elephant

genes. We first extracted the transcription start side and direction of each protein coding gene in the

Elephas maximus reference genome, then generated intervals for 1kb upstream of each gene. These

intervals were extracted using the samtools faidx program [66], and TFBS predicted using TFBStools [69] in

custom Rscript (TFBSTools_JASPAR2014.R). Identified TFBS were then cross referenced against filtered

mammoth deletions using bedtools intersect [68] to identify putative cis-regulatory deletions present in

woolly mammoths.

During the course of this project, a separate ancient DNA deletion pipeline was published [15]. We

also performed deletion prediction using the published pipeline using the same datasets above. We

examined the overlap between our two datasets using bedtools intersect [68].

4.3 – TP53 identification and primer design

We extracted and aligned the canonical TP53 locus as well as all 29 retrogenes (RTGs) from the

Elephas maximus reference genome (GCF_024166365.1). For the RTGs, we trimmed the alignment to

include only the ~1.1kb segment overlapping the exons in TP53. We then designed two primers to

specifically amplify the TP53 gene (Supplementary Table 2). For the RTGs, we designed seven forward

and four reverse primer variations, all amplifying the same locus, but containing all possible binding site

mutations to minimize amplification bias (Supplementary Table 2). These primers were made up to

10µM stock solutions and equal volumes of all forward and all reverse primers combined to produce

master forward and reverse stock solutions.

For all other deletions, we generated at least four primers flanking the region containing the

deletion ensuring similar melting temperatures across target sites, and that all primers were outside of

the region targeted by Cas9. For each locus, we tested all possible combinations of forward and reverse

primers and chose those that produced the cleanest and most reproducible amplifications

(Supplementary Table 2).

4.4 – Guide Design

We generated all possible guide RNAs (gRNAs) within TP53 from the start of first exon to the

end of the last exon using the generate_kmery.py script distributed as part of GuideScan2 [70]. All guides

were then screened for predicted off-targets against the entire *E. maximus* reference genome using

GuideScan2 with a maximum mismatch distance of 3 (-m 3), and with the option to output all guides (-t -

1). The output was then further filtered using a custom python script (Cas9_MMFiltering_V1_1.py) and

scoring matrix to filter potential gRNAs based on how many mismatches they possessed relative to

potential off-target sites and where along the spacer those mismatches occur. From the remaining

guides we selected two guides (Supplementary Table 1) that cut within exon 1 and between exon 2 and

3 respectively to generate TP53 knockouts as these disrupt the TAD1 domain and should interfere with

proper splicing resulting in a premature stop codon.

To design guides for the RTG knockouts, we generated all possible guides against the trimmed

RTG alignment for all 29 retrogenes using the generate_kmery.py script as before. We then screened all

guides for those that were predicted to have activity to the most possible targets and identified two

gRNAs that were each predicted to have activity against all but three retrogenes, which were only

targetable by either one of the two guides (Supplementary Table 2; Fig. 3). We then subsequently chose

one additional guide that targeted those three remaining retrogenes to ensure that each retrogene had

at least two gRNAs.

We also identified three genes that had consistent expression across our MSC and epithelial cell

line: FN1, EIF3I, and CD109. For each of these we designed all possible guides as above and filtered for

off-targets against the *E. maximus* reference genome using GuideScan2 and filtered as above. We then

selected four guides for each spaced roughly equally apart across a 1kb upstream region of each gene.

For all other deletions we extracted the *E. maximus* reference sequence ±25bp from the start or

end point of each deletion using samtools faidx [66] and generated all possible guides within those regions

using generate_kmery.py. We then filtered the automatically generated guides for off-targets using

GuideScan2 and the python script as above. In the case multiple guides were possible for each deletion

we chose one that would result in a double-stranded break closest to the original endpoint of each

deletion.

4.5 – Cell culturing and media

Fibroblast cells were grown in a custom media formulation with the final concentrations: 50%

FGM-2 (Lonza FBM Basal Media (CC-3131) supplemented with FGM-2 SingleQuots supplements (CC-

4126)); 20% heat-inactivated fetal bovine serum (ThermoFisher, A5670801); and 30% Mem Alpha

(Gibco, 12571063). Mesenchymal stem cells were grown in a custom media formulation with final

concentrations: 79% DMEM High Glucose + GlutaMax (ThermoFisher 10566016); 20% heat-inactivated

fetal bovine serum (ThermoFisher, A5670801); and 1% MEM Non-Essential Amino Acids Solution

(ThermoFisher 11140050). Epithelial cells were grown in a custom media formulation with final

concentrations: EGM-2 (Lonza Basal Medium (CC-3156) supplemented with EGMTM-2 SingleQuotsTM

Supplements (CC-4176)); 40% DMEM High Glucose + GlutaMax (ThermoFisher 10566016); and 10%

heat-inactivated fetal bovine serum (ThermoFisher, A5670801). Cells were grown in an incubator at 37°C

with 5% CO_2.

4.6 – Cas9 RNP Electroporation

All gRNAs were ordered from Synthego and resuspended to 100µM in 1X TE buffer. Cas9

nuclease was ordered from IDT (catalog # 10007807). All nucleofections were conducted using the Lonza

P4 Primary Cell 96-well Kit (catalog # V4SP-4096) using codes CA-137 (fibroblasts) or EN-150 (epithelial

cells), or the P3 Primary Cell 96-well Kit (catalog # V4SP-3096) using code CA-137 (MSCs).

For the TFBS, synthetic, and large-mammoth deletions, approximately 100-200k cells were used

for each 20µl transfection reaction, with a 3µM final concentration of each guide (i.e. both upstream

and downstream of the deletion) and 1µM of Cas9 nuclease per guide (i.e. a 3:1 ratio of gRNA:Cas9).

RNPs were pre-complexed in nucleofection solution for 20 minutes at room temperature then

electroporated into cells using the above codes.

We conducted pilot studies for the seven identified TFBS deletions upstream of genes that

showed consistent expression in our MSC line, although only one guide was used for deletions upstream

of LOC126074853 and LOC126068891 due to the small size of these deletions. We also conducted pilot

studies using all three synthetic deletion genes (FN1, CD109, EIF3I), as well as for the six smallest

intragenic and seven smallest intergenic large mammoth-specific deletions. Deletions were carried out

alongside negative control containing just Cas9 and/or a non-targeting gRNA RNP which has no

significant homology to the *E. maximus* genome. All experimental conditions for pilot work were

conducted in duplicate and in MSCs. We subsequently chose a subset of these deletions which

generated a high number of DEGs for our final analysis. These deletions were done as above, except

with four replicates each and accompanied by 3-4 negative controls using a non-targeting gRNA. For

Deletions 3, 51, and 89 we also attempted to introduce these deletions into fibroblast and epithelial

cells, however the epithelial cells failed to recover from the nucleofections and were ultimately

discarded.

TP53 deletions were done as above, except with 400-500k cells per transfection. Cells were

transfected with either both TP53 gRNAs, all three RTG gRNAs, both TP53 and RTG gRNAs ("All"

condition), or a non-targeting gRNA (Supplementary Table 1). For the All nucleofection, gRNAs were first

combined and then concentrated using a Savant SpeedVac DNA 120 Concentrator (Thermo Scientific),

before being resuspended in premade nucleofector and supplement solution. Additionally, due to the

presence of possible TP53 mutations that accumulate with prolonging passaging, a second separate

nucleofection was conducted as above prior to the Mitomycin C stressed RNA-seq. In both cases, all

treatment conditions were conducted using three biological replicates. Due to their greater robustness,

their ease of culture and use, and the availability of younger passage cells, we elected to conduct these

experiments only in our fibroblast line.

Following nucleofection cells were immediately transferred into 6-well plates containing

prewarmed media and allowed to recover for 4 days. Media was changed 24 hours post nucleofection

and every 2-3 days thereafter.

4.7 – Editing Efficiency

During the first passage (approximately 4 days post nucleofection), about 10% of the cells were

collected and DNA extracted using the DNeasy Blood and Tissue Kit (Qiagen) using the manufacturer's

recommended protocol. For the TP53-editing extractions we amplified both the TP53 locus spanning the

expected knockout deletion, and the retrogene loci. For all other extractions we amplified the locus

spanning the targeted deletion and examined these loci across the negative controls as well. We

preformed 25µL PCR reactions with final concentrations of 1X Phusion Hot Start Flex 2X Master Mix and

500nM of each primer, using between 1-3µL of template. Cycling conditions were: 98°C for 3 minutes;

12 cycles of 98°C for 15 seconds, then 69°C for 60 seconds; 25 cycles of 98°C for 15 seconds, 67°C for 30

seconds, then 72°C for 45 seconds; followed by a final extension at 72°C for 5 minutes. Resulting PCR

amplicons were purified over MinElute PCR Purification columns (Qiagen) using the recommended

protocol and visualized on E-gel EX 2% Agarose gels (Invitrogen) to confirm the presence or absence of

any large deletions. We examined the relative fluorescence of our products using the Image Lab 6.1 (Bio-

Rad). We then calculated a minimum editing efficiency (the relative fluorescence of our expected

deleted product, relative to everything else in the lane) and a maximum editing efficiency (the relative

fluorescence of all non-wild-type bands within each lane).

To confirm efficiency estimates and examine RTG deletion distribution in our TP53-editing

experiments, we sent each of our TP53 and RTG amplicon pools for nanopore sequencing using the

Premium PCR service at Primordium to get full length sequences of our DNA fragments. For each

sample, raw reads were first filtered using a custom python script (FastqFilter.py) to between the

maximum and minimum expected sizes +/- 10% respectively. Mapping was carried out using BBMap [71]

using default parameters against either the TP53 amplicon, an RTG consensus amplicon, or a multi-fasta

with 26 RTGs (RTG amplicons with identical sequences outside of the editing region collapsed).

To calculate editing efficiency, we used a custom python script (CigarDel.py) which uses a region

file containing the deletion regions of interest, a minimum deletion proportion (i.e. the proportion of

bases mapping to the deleted region that need to be missing), and the CIGAR string of each read to

evaluate the presence or absence of various deletions in the data. For TP53 we analyzed the presence of

the expected deletion if Cas9 induced a double-strand break at both sgRNA targeting sites. For the RTG

we examined the presence of any of the three possible deletions between two guides. All analyses were

carried out with a minimum deletion proportion of 0.9.

To examine the distribution of deletions at each locus we identified the names of reads

containing deletions and extracted those that mapped to each locus. We then cross-referenced this with

the deletions identified against the RTG consensus to obtain read distributions for reads mapped to all

RTGs or the collapsed RTG loci.

4.8 – Bulk RNA-seq

Approximately 300-500k cells were collected, flash frozen on dry ice, and shipped to Genewiz

for bulk RNA sequencing within ten days of transfection (TP53) or 2-3 weeks (all other deletions).

Expression of transcripts was done using Salmon (v1.8.0 [72]) using a custom index consisting of all mRNAs

and non-coding RNAs annotated within the *E. maximus* reference genome (GCF_024166365.1), with all

29 TP53 retrogene sequences added. The salmon index was generated using the keepDuplicates flag to

avoid collapsing any RTGs. Salmon quant files were analyzed using DESeq2 [73] as part of a custom Rscript

to analyze samples in a pairwise fashion (i.e. one set of biological replicates against the controls at a

time). Differentially expressed genes (DEGs) were identified with an adjusted p value of less than 0.05

(padj < 0.05). No minimum log2 fold change was applied for initial analysis of TP53 pathway genes. A

minimum absolute log2 fold change of 1 was used for all other analyses. Venn diagrams and DEG

overlap was generated using a web based custom venn diagram tool

(https://bioinformatics.psb.ugent.be/webtools/Venn/), and pathway enrichment preformed using

GeneCodis4 [74].

4.9 – Retrogene expression in public datasets

To ensure the expression patterns we observed were not tissue/cell type specific, we examined

the expression of our retrogenes in some preliminary shared data from mesenchymal stem cells and

epithelial cells. These cells contained deletions generated using the same guides as in this study, but the

epithelial cells were also subject to various small molecule treatments. We also downloaded public RNA-

seq datasets for Asian elephant ovary (SRX15554240), salivary glands (SRX15554239), lymph nodes

(SRX15554238), thyroid (SRX15554237), lung (SRX15554236), and lymphocytes (SRX1423033), and

analyzed it as above.

4.10 – Depth plots

To examine where reads from each of the three RTGs showing repeated expression were

originating, we mapped the data for our datasets and the public datasets back to the Asian elephant

reference genome using STAR [75]. We then extracted read depths along the full annotated length of RTGs

LOC126068247, LOC126068267, and LOC126068248 using samtools depth [76]. For our dataset we

averaged expression values within biological replicates and plotted the data in Microsoft Excel.

4.11 – Maximum likelihood phylogeny

We download African elephant retrogene 9 (KF715863.1) to investigate a possible relationship

between this retrogenes and the Asian elephant retrogenes. We then aligned the retrogene to the Asian

elephant TP53 CDS and truncated the RTG 9 sequence to contain only the homologous sequence to

TP53. We then aligned the truncated RTG 9 sequence, Asian elephant TP53, and all 29 Asian elephant

retrogenes using muscle (v5.1) [77]. We generated a maximum likelihood phylogeny using IQ-Tree [78], using

ModelFinder to identify the best supported substitution model [79], and 100 bootstraps (-b 100).

Supplementary References: [12,65,71,72,80–85]

5. Author contributions

EK and GMC conceived the study with feedback from EM and AAT. EK and NB designed and

conducted all wet-lab experiments with feedback and help from EM, AAT, and LL. EK and LL completed

all bioinformatic analyses. AAT provided additional RNA-seq data for epithelial cell lines. EK wrote the

first draft of the manuscript. All authors helped in the revision of subsequent drafts.

6. Data availability

Bulk RNA-seq data for all mammoth deletions as well as TP53 conditions with and without

mitomycin was deposited on NCBI (BioProject Accessions: PRJNA1147676 and PRJNA1217465).

Bioinformatic scripts and tools created for this work are available at:

https://github.com/EmilKarpinski/RTG_TP53_MammothDel_Analysis.

7. Declaration of competing interests

For a complete list of G.M.C.'s financial interests, please visit

http://arep.med.harvard.edu/gmc/tech.html. All other authors declare no other conflicts of interest.

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600   **References**

601   1.   Mack, K. L. & Nachman, M. W. Gene Regulation and Speciation. *Trends in Genetics* **33**, 68–80
602   (2017).

603   2.   Romero, I. G., Ruvinsky, I. & Gilad, Y. Comparative studies of gene expression and the evolution
604   of gene regulation. *Nat Rev Genet* **13**, 505–516 (2012).

605   3.   Hu, L. *et al.* Arctic introgression and chromatin regulation facilitated rapid Qinghai-Tibet Plateau
606   colonization by an avian predator. *Nat Commun* **13**, 6413 (2022).

607   4.   Lin, Z. *et al.* Biological adaptations in the Arctic cervid, the reindeer (*Rangifer tarandus*). *Science*
608   *(1979)* **364**, (2019).

609   5.   Mack, K. L., Ballinger, M. A., Phifer-Rixey, M. & Nachman, M. W. Gene regulation underlies
610   environmental adaptation in house mice. *Genome Res* **28**, 1636–1645 (2018).

611   6.   Shoshani, J. Understanding proboscidean evolution: a formidable task. *Trends Ecol Evol* **13**, 480–
612   487 (1998).

613   7.   Larramendi, A. Proboscideans: Shoulder Height, Body Mass and Shape. *Acta Palaeontol Pol* **61**,
614   537–574 (2015).

615   8.   Boeskorov, G. G. *et al.* Adaptation of the woolly mammoth Mammuthus primigenius
616   (Blumenbach, 1799) to habitat conditions in the glacial period. *Contemp Probl Ecol* **9**, 544–553
617   (2016).

618   9.   van der Valk, T. *et al.* Million-year-old DNA sheds light on the genomic history of mammoths.
619   *Nature* **591**, 265–269 (2021).

620   10.   Lynch, V. J. *et al.* Elephantid Genomes Reveal the Molecular Bases of Woolly Mammoth
621   Adaptations to the Arctic. *Cell Rep* **12**, 217–228 (2015).

622   11.   Peto, R., Roe, F. J., Lee, P. N., Levy, L. & Clack, J. Cancer and ageing in mice and men. *Br J Cancer*
623   **32**, 411–426 (1975).

624   12.   Sulak, M. *et al.* TP53 copy number expansion is associated with the evolution of increased body
625   size and an enhanced DNA damage response in elephants. *Elife* **5**, (2016).

626   13.   Abegglen, L. M. *et al.* Potential Mechanisms for Cancer Resistance in Elephants and Comparative
627   Cellular Response to DNA Damage in Humans. *JAMA* **314**, 1850 (2015).

628   14.   Preston, A. J. *et al.* Elephant TP53-RETROGENE 9 induces transcription-independent apoptosis at
629   the mitochondria. *Cell Death Discov* **9**, 66 (2023).

630   15.   van der Valk, T. *et al.* Evolutionary consequences of genomic deletions and insertions in the
631   woolly mammoth genome. *iScience* **25**, 104826 (2022).

632   16.   Endoh-Yamagami, S. *et al.* A mutation in the pericentrin gene causes abnormal interneuron
633   migration to the olfactory bulb in mice. *Dev Biol* **340**, 41–53 (2010).

17. Denu, R. A. *et al.* Fibroblasts and Mesenchymal Stromal/Stem Cells Are Phenotypically
Indistinguishable. *Acta Haematol* **136**, 85–97 (2016).

18. Närhi, K. *et al.* Sostdc1 defines the size and number of skin appendage placodes. *Dev Biol* **364**,
149–161 (2012).

19. Yoon, S.-Y. & Detmar, M. Sostdc1 Secreted from Cutaneous Lymphatic Vessels Acts as a Paracrine
Factor for Hair Follicle Growth. *Curr Issues Mol Biol* **44**, 2167–2174 (2022).

20. Cunningham, L. L., Matsui, J. I., Warchol, M. E. & Rubel, E. W. Overexpression of *Bcl-2* prevents
neomycin-induced hair cell death and caspase-9 activation in the adult mouse utricle *in vitro*. *J
Neurobiol* **60**, 89–100 (2004).

21. Schlake, T. Segmental Igfbp5 expression is specifically associated with the bent structure of zigzag
hairs. *Mech Dev* **122**, 988–997 (2005).

22. Khnykin, D., Miner, J. H. & Jahnsen, F. Role of fatty acid transporters in epidermis.
Dermatoendocrinol **3**, 53–61 (2011).

23. Owada, Y. *et al.* Altered Water Barrier Function in Epidermal-Type Fatty Acid Binding Protein-
Deficient Mice. *Journal of Investigative Dermatology* **118**, 430–435 (2002).

24. Vanderwolf, K., Kyle, C. & Davy, C. A review of sebum in mammals in relation to skin diseases,
skin function, and the skin microbiome. *PeerJ* **11**, e16680 (2023).

25. Repin, V. E., Taranov, O. S., Ryabchikova, E. I., Tikhonov, A. N. & Pugachev, V. G. Sebaceous
Glands of the Woolly Mammoth, Mammothus primigenius Blum.: Histological Evidence. *Doklady
Biological Sciences* **398**, 382–384 (2004).

26. Martin, T. G. & Leinwand, L. A. Molecular regulation of reversible cardiac remodeling: lessons
from species with extreme physiological adaptations. *Journal of Experimental Biology* **227**,
(2024).

27. Keen, A. N., Klaiman, J. M., Shiels, H. A. & Gillis, T. E. Temperature-induced cardiac remodeling in
fish. *Journal of Experimental Biology* **220**, 147–160 (2016).

28. Salsberg, C. A. Resurrecting the woolly mammoth: science, law, ethics, politics, and religion. *Stan.
Tech. L. Rev.* (2000).

29. Grego-Bessa, J. *et al.* Nrg1 Regulates Cardiomyocyte Migration and Cell Cycle in Ventricular
Development. *Circ Res* **133**, 927–943 (2023).

30. Ushikoshi, H. *et al.* Local overexpression of HB-EGF exacerbates remodeling following myocardial
infarction by activating noncardiomyocytes. *Laboratory Investigation* **85**, 862–873 (2005).

31. Cordero, P. *et al.* Pathologic gene network rewiring implicates PPP1R3A as a central regulator in
pressure overload heart failure. *Nat Commun* **10**, 2760 (2019).

32. Krumbein, M. *et al.* RNA binding protein IGF2BP2 expression is induced by stress in the heart and
mediates dilated cardiomyopathy. *Commun Biol* **6**, 1229 (2023).

33.   Wang, J. *et al.* Cardiomyopathy Associated with Microcirculation Dysfunction in Laminin α4
670   Chain-deficient Mice. *Journal of Biological Chemistry* **281**, 213–220 (2006).

671   34.   Jiang, Z. *et al.* SEC14L3 knockdown inhibited clear cell renal cell carcinoma proliferation,
672   metastasis and sunitinib resistance through an SEC14L3/RPS3/NFκB positive feedback loop.
673   *Journal of Experimental & Clinical Cancer Research* **43**, 288 (2024).

674   35.   Thyboll, J. *et al.* Deletion of the Laminin α4 Chain Leads to Impaired Microvessel Maturation. *Mol*
675   *Cell Biol* **22**, 1194–1202 (2002).

676   36.   Wade, G., McGahee, A., Ntambi, J. M. & Simcox, J. Lipid Transport in Brown Adipocyte
677   Thermogenesis. *Front Physiol* **12**, (2021).

678   37.   Eguchi, J. *et al.* Transcriptional Control of Adipose Lipid Handling by IRF4. *Cell Metab* **13**, 249–259
679   (2011).

680   38.   Dai, N. *et al.* IGF2BP2/IMP2-Deficient Mice Resist Obesity through Enhanced Translation of Ucp1
681   mRNA and Other mRNAs Encoding Mitochondrial Proteins. *Cell Metab* **21**, 609–621 (2015).

682   39.   Song, B., Scheuner, D., Ron, D., Pennathur, S. & Kaufman, R. J. Chop deletion reduces oxidative
683   stress, improves β cell function, and promotes cell survival in multiple mouse models of diabetes.
684   *Journal of Clinical Investigation* **118**, 3378–3389 (2008).

685   40.   Wang, B. *et al.* Identification and characterization of NYGGF4, a novel gene containing a
686   phosphotyrosine-binding (PTB) domain that stimulates 3T3-L1 preadipocytes proliferation. *Gene*
687   **379**, 132–140 (2006).

688   41.   Zhang, Y. *et al.* Targeting nuclear receptor NR4A1–dependent adipocyte progenitor quiescence
689   promotes metabolic adaptation to obesity. *Journal of Clinical Investigation* **128**, 4898–4911
690   (2018).

691   42.   Wang, S.-S. *et al.* Early growth response 2 (Egr2) plays opposing roles in committing C3H10T1/2
692   stem cells to adipocytes and smooth muscle-like cells. *Int J Biochem Cell Biol* **45**, 1825–1832
693   (2013).

694   43.   Tran, M. T. N. *et al.* MafB deficiency accelerates the development of obesity in mice. *FEBS Open*
695   *Bio* **6**, 540–547 (2016).

696   44.   Xiao, Z., Chu, Y. & Qin, W. IGFBP5 modulates lipid metabolism and insulin sensitivity through
697   activating AMPK pathway in non-alcoholic fatty liver disease. *Life Sci* **256**, 117997 (2020).

698   45.   Herijgers, N. *et al.* Effect of human scavenger receptor class A overexpression in bone marrow-
699   derived cells on lipoprotein metabolism and atherosclerosis in low density lipoprotein receptor
700   knockout mice. *J Lipid Res* **41**, 1402–1409 (2000).

701   46.   Thompson, E. M. & Stoker, A. W. A Review of DUSP26: Structure, Regulation and Relevance in
702   Human Disease. *Int J Mol Sci* **22**, 776 (2021).

703   47.   Hung, Y.-H., Carreiro, A. L. & Buhman, K. K. Dgat1 and Dgat2 regulate enterocyte triacylglycerol
704   distribution and alter proteins associated with cytoplasmic lipid droplets in response to dietary

fat. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1862**, 600–614
(2017).

48.   Betters, J. L. & Yu, L. NPC1L1 and cholesterol transport. *FEBS Lett* **584**, 2740–2747 (2010).

49.   Cai, W. *et al.* The kainate receptor GluK2 mediates cold sensing in mice. *Nat Neurosci* **27**, 679–
688 (2024).

50.   Esselstyn, J. A., Oliveros, C. H., Swanson, M. T. & Faircloth, B. C. Investigating Difficult Nodes in
the Placental Mammal Tree with Expanded Taxon Sampling and Thousands of Ultraconserved
Elements. *Genome Biol Evol* **9**, 2308–2321 (2017).

51.   Marzec, K. A., Lin, M. Z., Martin, J. L. & Baxter, R. C. Involvement of p53 in insulin-like growth
factor binding protein-3 regulation in the breast cancer cell response to DNA damage.
Oncotarget **6**, 26583–26598 (2015).

52.   Grimberg, A. *et al.* p53-Dependent and p53-Independent Induction of Insulin-Like Growth Factor
Binding Protein-3 by Deoxyribonucleic Acid Damage and Hypoxia. *J Clin Endocrinol Metab* **90**,
3568–3574 (2005).

53.   Hermeking, H. The 14-3-3 cancer connection. *Nat Rev Cancer* **3**, 931–943 (2003).

54.   Hermeking, H. *et al.* 14-3-3σ Is a p53-Regulated Inhibitor of G2/M Progression. *Mol Cell* **1**, 3–11
(1997).

55.   Huang, R. *et al.* The NCATS BioPlanet – An Integrated Platform for Exploring the Universe of
Cellular Signaling Pathways for Toxicology, Systems Biology, and Chemical Genomics. *Front
Pharmacol* **10**, (2019).

56.   Dey, G. *et al.* Signaling network of Oncostatin M pathway. *J Cell Commun Signal* **7**, 103–108
(2013).

57.   Moldovan, G.-L. & D'Andrea, A. D. How the Fanconi Anemia Pathway Guards the Genome. *Annu
Rev Genet* **43**, 223–249 (2009).

58.   Du, W. & Searle, J. The Rb Pathway and Cancer Therapeutics. *Curr Drug Targets* **10**, 581–589
(2009).

59.   de Visser, K. E. & Joyce, J. A. The evolving tumor microenvironment: From cancer initiation to
metastatic outgrowth. *Cancer Cell* **41**, 374–403 (2023).

60.   Guha, S. & Bhaumik, S. R. Transcription-coupled DNA double-strand break repair. *DNA Repair
(Amst)* **109**, 103211 (2022).

61.   Sonawane, A. R. *et al.* Understanding Tissue-Specific Gene Regulation. *Cell Rep* **21**, 1077–1088
(2017).

62.   Tollis, M. *et al.* Elephant Genomes Reveal Accelerated Evolution in Mechanisms Underlying
Disease Defenses. *Mol Biol Evol* **38**, 3606–3620 (2021).

63. Li, Z. *et al.* Establishment of Bactrian Camel Induced Pluripotent Stem Cells and Prediction of
Their Unique Pluripotency Genes. *Int J Mol Sci* **24**, 1917 (2023).

64. Appleton, E. *et al.* Derivation of elephant induced pluripotent stem cells. *bioRxiv* (2024)
doi:10.1101/2024.03.05.583606.

65. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform.
Bioinformatics **25**, 1754–1760 (2009).

66. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079
(2009).

67. Neph, S. *et al.* BEDOPS: high-performance genomic feature operations. *Bioinformatics* **28**, 1919–
1920 (2012).

68. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features.
Bioinformatics **26**, 841–842 (2010).

69. Tan, G. & Lenhard, B. TFBSTools: an R/bioconductor package for transcription factor binding site
analysis. *Bioinformatics* **32**, 1555–1556 (2016).

70. Schmidt, H. *et al.* Genome-wide CRISPR guide RNA design and specificity analysis with
GuideScan2. (2022) doi:10.1101/2022.05.02.490368.

71. Bushnell, B. BBMap. http://sourceforge.net/projects/bbmap/ (2022).

72. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon provides fast and bias-
aware quantification of transcript expression. *Nat Methods* **14**, 417–419 (2017).

73. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-
seq data with DESeq2. *Genome Biol* **15**, 550 (2014).

74. Garcia-Moreno, A. *et al.* Functional Enrichment Analysis of Regulatory Elements. *Biomedicines* **10**,
590 (2022).

75. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

76. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079
(2009).

77. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput.
Nucleic Acids Res **32**, 1792–1797 (2004).

78. Nguyen, L.-T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: A Fast and Effective
Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol Biol Evol* **32**, 268–274
(2015).

79. Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A. & Jermiin, L. S. ModelFinder:
fast model selection for accurate phylogenetic estimates. *Nat Methods* **14**, 587–589 (2017).

80. Lee, Y.-J., Park, S.-J., Ciccone, S. L. M., Kim, C.-R. & Lee, S.-H. An in vivo analysis of MMC-induced
DNA damage and its repair. *Carcinogenesis* **27**, 446–453 (2006).

81.   Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

82.   Fukuda, T. *et al.* Expression of human cell cycle regulators in the primary cell line of the African
savannah elephant (loxodonta africana) increases proliferation until senescence, but does not
induce immortalization. *In Vitro Cell Dev Biol Anim* **52**, 20–26 (2016).

83.   Pearson, V. R. *et al.* Identification of African Elephant Polyomavirus in wild elephants and the
creation of a vector expressing its viral tumor antigens to transform elephant primary cells. *PLoS*
One **16**, e0244334 (2021).

84.   Housden, B. E. *et al.* Identification of potential drug targets for tuberous sclerosis complex by
synthetic screens combining CRISPR-based knockouts with RNAi. *Sci Signal* **8**, (2015).

85.   Munroe, R. & Schimenti, J. Mutagenesis of Mouse Embryonic Stem Cells with
Ethylmethanesulfonate. in 131–138 (2009). doi:10.1007/978-1-59745-471-1_7.

Figure 1. Experimental Design. A. Trends in proboscidean evolution include a general increase in body
size, and adaptations to various environments including warm tropical grasslands and cold arctic tundra.
B. We engineered mammoth specific or synthetic cis-regulatory deletions, large mammoth-specific
deletions in both intra and intergenic regions, as well as deletions within TP53 and/or all TP53 RTGs
using Cas9 RNPs within various Asian elephant cells in culture, before examining their effects via RNA-
seq.

Figure 2. Mammoth deletion efficiency and effects. A. Editing efficiency observed for each deletion in both MSCs and fibroblasts estimated using gel electrophoresis. **B.** DEG overlap between MSCs and fibroblasts for each of the three large mammoth-specific noncoding deletions. **C.** A selection of pathways potentially related to arctic adaptation. Bars are colored blue/red for upregulated and downregulated genes respectively. The database and key genes are listed beside each bar. A vertical dashed line indicates a <0.05 significance level.

Figure 3. TP53 Editing. A. Schematic showing an alignment of Asian elephant TP53 and a retrogene (RTG) consensus, with domains labelled
based on homology to human TP53. Exons are shown in yellow, with introns indicated by black lines. Missing sequence is indicated with a
dashed line in the RTGs. Additionally, we've included the positions of deletions greater than 3bp in the RTG consensus, and the approximate
locations of stop codons observed across the 29 Asian elephant retrogenes. The locations and directions of guides are shown for both TP53 and
the retrogenes. **B.** Predicted guide activity against each of the 29 Asian elephant retrogenes, with green indicating the guide is expected to be

808   functional against that target and red indicating non-functionality. **C.** Observed editing efficiency of the expected deletions based on nanopore

809   data. **D.** The frequency of deletions observed in all reads mapped against the RTG consensus across different treatment conditions. **E.** The

810   frequency of each deletion across the individual Asian elephant retrogenes as a proportion of all deletion-containing reads mapped to that locus,

811   or all mapped read (**F**). Amplicons which were identical across the non-edited regions were collapsed as they are bioinformatically

812   undistinguishable. Error bars represent standard deviation in all plots.

Figure 4. Expression of TP53 pathway genes. Log2 fold changes observed for genes immediately downstream of TP53 in the p53 signaling pathway (KEGG pathway hsa04115). Crosses indicate that no significant difference in expression was observed for that gene in a given condition. Genes in italics have an absolute log2 fold change of < 1 and were filtered out of enrichment analyses. Only downstream genes with a significant difference in at least one condition are shown.

Figure 5. Pathway enrichment. A. Differentially expressed gene (DEG) overlap observed between
treatment conditions for cells in normal media or exposed to 10μM mitomycin C. Genes were filtered
for a minimum absolute log2 fold change greater than 1. **B.** BioPlanet pathway enrichment for the 763
DEGs common to all three conditions in the Mitomycin C treatment cells. **C.** DEG overlap between TP53
KO and RTG KO cells in normal media or exposed to 10μM Mitomycin C. **D.** BioPlanet pathway

825   enrichment of unique DEGs in TP53 KO Mitomycin C treated cells. **E.** BioPlanet pathway enrichment of
826   unique DEGs in RTG KO Mitomycin C treated cells. **F.** Pathway/gene cluster analysis for DEGs in the RTG
827   KO Mitomycin C treated cells. ECM related pathways are colored green and labelled. Pathway clusters
828   are scaled by their p-value.