A Polymorphism in IRF4 Affects Human Pigmentation through a Tyrosinase-Dependent MITF/TFAP2A Pathway

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SUMMARY

Sequence polymorphisms linked to human diseases and phenotypes in genome-wide association studies often affect noncoding regions. A SNP within an intron of the gene encoding Interferon Regulatory Factor 4 (IRF4), a transcription factor with no known role in melanocyte biology, is strongly associated with sensitivity of skin to sun exposure, freckles, blue eyes, and brown hair color. Here, we demonstrate that this SNP lies within an enhancer of IRF4 transcription in melanocytes. The allele associated with this pigmentation phenotype impairs binding of the TFAP2A transcription factor that, together with the melanocyte master regulator MITF, regulates activity of the enhancer. Assays in zebrafish and mice reveal that IRF4 cooperates with MITF to activate expression of Tyrosinase (TYR), an essential enzyme in melanin synthesis. Our findings provide a clear example of a noncoding polymorphism that affects a phenotype by modulating a developmental gene regulatory network.

INTRODUCTION

Human pigmentation is a complex process involving melanocytes that synthesize the pigment melanin in melanosomes, cell organelles that are transferred to neighboring keratinocytes, where they form a cap over nuclei, thus protecting them from negative effects of UV radiation (UVR). Pigmentation is not only one of the most distinguishing features of humans but also serves an important protective role. In humans, pigmentation decreases with increasing distance from the equator, presumably due to a balance between the pressure to optimize the amount of available UVR for the generation of vitamin D3 and the protection from UVR-mediated damage, which may result in increased risk of cutaneous malignancies. This has led to positive selection for less-pigmented skin, hair, and eyes in areas distant from the equator. The major difference between dark- and light-skinned individuals is due to differences in the number, size, and density of the melanin-containing melanosomes; the number of melanocytes is roughly the same (reviewed in Sturm, 2009).

The genetics of pigmentation is complex and involves several genes and pathways. It has been best characterized in the mouse where 171 of nearly 400 loci implicated in pigmentation have been functionally characterized (http://www.espcr.org/micemut/). These pigmentation genes affect various steps in the formation of melanocytes from the neural crest (e.g., *Mgf*, *Kit*, and *Mitf*), the generation of components of melanosomes and pigment (e.g., *pMel17/Silver* and *Tyr*), or the transport of melanosomes along microtubules in the dendrites before delivery to adjacent keratinocytes (e.g., *MyoVa*, *Rab27*, and *Mlph*). Most genes involved in pigmentation are transcriptionally regulated by the bHLHZip transcription factor MITF, the master regulator of melanocytes (reviewed in Steingrímsson et al., 2004).

Genome-wide association studies (GWASs) have identified several SNPs involved in human pigmentation, including SNPs on chromosome 6 near the *DUSP22*, *Interferon Regulatory Factor 4* (*IRF4*), and *EXOC2* genes, none of which was previously implicated in pigmentation (Sulem et al., 2007, 2008; Han et al., 2008). The SNP showing the strongest association is located in intron 4 of *IRF4* (Han et al., 2008). IRF4 belongs to the Interferon Regulatory Factors (IRFs), a wing-helix-turn-helix family of transcription factors that regulate interferon (IFN)-inducible genes (Paun and Pitha, 2007). Although IRF4 does not depend on IFN stimulation, it binds to the IFN-stimulated response element (ISRE) within IFN-responsive genes (Grossman et al., 1996; Escalante et al., 1998). *Irf4* mutant mice completely lack germinal centers and plasma cells and show severely reduced immunoglobulin levels in the serum; no defects in pigmentation were reported (Mittrücker et al., 1997). In a subset of multiple patients with myeloma, *IRF4* is translocated downstream of the immunoglobulin heavy-chain regulatory regions (Iida et al., 1997; Tsuboi et al., 2000). Interestingly, in this disease, *IRF4* acts as a lineage survival oncogene regardless of translocation status (Shaffer et al., 2008). Polymorphisms in the *IRF4* gene are associated with chronic lymphocytic leukemia (CLL) (Di Bernardo et al., 2008) and with nonhematopoietic diseases, including celiac disease (Dubois et al., 2010), and progressive supranuclear palsy (Höglinger et al., 2011). Finally, IRF4 was recently shown to be critical for transcriptional response to nutrient availability in adipocytes (Eguchi et al., 2011).

A few reports have linked *IRF4* to pigment cells. *IRF4* is expressed in melanocytes in the skin and in the G361 melanoma cell line (Grossman et al., 1996) as well as in most melanomas (Sundram et al., 2003). Importantly, IRF4 is associated with human pigmentation (Sulem et al., 2007; Han et al., 2008), and IRF4 expression correlates with MITF expression in melanoma cells (Hoek et al., 2008). Here, we show that *IRF4* is involved in pigmentation, that the melanocyte master regulator MITF activates expression of the *IRF4* gene, and that this activation depends on the presence of the transcription factor activator protein 2α (TFAP2A). A naturally occurring sequence variant associated with human pigmentation overlaps the TFAP2A-binding site, impairs binding by this transcription factor, and consequently, lowers induction of IRF4 expression. Together, the MITF and IRF4 proteins cooperatively activate expression of the gene encoding the pigmentation enzyme Tyrosinase (TYR). This activation depends on MITF- and IRF4-binding sites in the TYR promoter. Thus, we have established a direct link between a polymorphism in an intron in the *IRF4* gene and reduced expression of an enzyme essential for pigmentation.

RESULTS

Fine Mapping of the IRF4 Locus Implicates rs12203592 as a Functional Variant Affecting Pigmentation

Several sequence variants in the *IRF4* locus have been associated with freckling, sun sensitivity, eye and hair color, and with nevus counts, with rs12203592-[T/C] showing the strongest association (Sulem et al., 2007; Han et al., 2008; Duffy et al., 2010a). The rs12203592-T minor allele is most common in individuals of European descent; it is not seen in sub-Saharan Africans or in East Asians (see Figure S1 available online). Analysis of sequenced vertebrate species shows that this position is occupied by C, suggesting that it is the ancestral allele.

In order to fine map the pigmentation trait associations at the *IRF4* locus, we used whole-genome sequencing data derived from 2,230 Icelanders sequenced to an average coverage of at least 10×. This yielded approximately 38.5 million SNP and small indel (insertion or deletion) variants genome wide, 16,280 of which were located in the region around *IRF4* (0–1 Mb on chromosome 6). Using imputation assisted by long-range haplotype phasing (Kong et al., 2008, 2009; Holm et al., 2011), we determined the genotypes of these 16,280 variants in 95,085 individuals who had been typed using Illumina SNP chips. We then tested each variant for association with eye color, hair color, freckling, and sun sensitivity, observing numerous significant signals (Figure 1). The strongest signals in the *IRF4* region came from the association of rs12203592-T with presence of freckles, brown hair, and high sensitivity of skin to sun exposure (Figure 1; Table S1). The second-most significant variant association was for rs62389424-A with freckles (p = 1.1 × 10^{-81}), nearly 40 orders of magnitude less significant than the corresponding signal from rs12203592-T (p = 2.0 × 10^{-120}). The rs62389424 variant is correlated with rs12203592 with an r^2 of 0.65. Two other SNPs in the region have r^2 values in excess of 0.2 with rs12203592, and they also gave association signals with similar phenotypes (Table S2). When, in a multivariate analysis, the associations were conditioned on the effect of rs12203592, the signals from the three correlated SNPs became nonsignificant (Table S2). Indeed, once rs12203592 was taken into account in the multivariate analysis, no variant in the *IRF4* region retained a significant signal after Bonferroni correction for the number of tests (Figure 1). We note in this context that the signal from the originally reported pigmentation variant at this locus, rs1540771-T (Sulem et al., 2007), was also captured by rs12203592 (Table S2). Thus, the associations at the *IRF4* locus with freckles, hair color, sun sensitivity, and eye color could all be accounted for by rs12203592, and no other variant detected by sequencing explains the effect with a similar level of significance.

Intron 4 of IRF4 Contains a Melanocyte-Specific Enhancer Element

rs12203592 is located in intron 4 of the IRF4 gene, suggesting that this SNP might alter the function of a *cis*-regulatory element. Data from the ENCODE consortium (Thurman et al., 2012) show that rs12203592 overlaps a peak of DNase I hypersensitivity
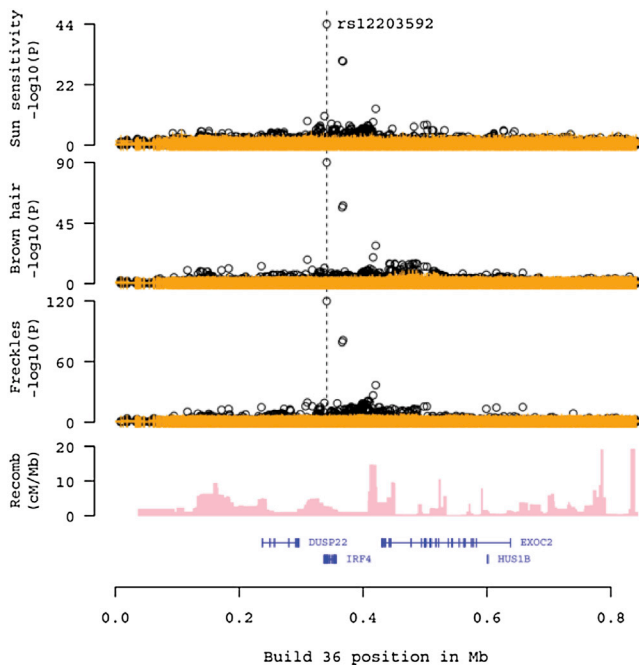
Figure 1. Association of SNPs in the IRF4 Region with Pigmentation Phenotypes

Association with pigmentation traits of variants in the IRF4 region (0–1 Mb on chromosome 6) was determined by whole-genome sequencing and imputation into SNP chip-typed individuals. Black points show the unadjusted association values; yellow points show association values adjusted for the effect of rs12203592. The x axis is the genomic coordinate (hg18 Build 36). The y axes are the association-$\log_{10}$ p values for sun sensitivity (yes/no, upper panel), brown versus blond hair color (second panel), and freckling (yes/no, third panel). The position of rs12203592 is indicated. The fourth panel shows the local recombination (Recomb) rates in cM/Mb. The lowest panel indicates the locations of known genes in the region, from UCSC. See also Table S1 and Figure S1.

(HS), a property of active regulatory elements, in human primary melanocytes and three human melanoma lines (Figure 2A). Moreover, rs12203592 does not overlap a DNase I-HS peak in 158 of 165 (96%) non-melanocyte-derived cell types examined by ENCODE, suggesting that the regulatory activity in this region is specific to the melanocyte lineage. In a subset of 49 ENCODE cell types assayed by the Crawford group (Duke University) for both DNase I-HS and global gene expression, *IRF4* is expressed at high levels only in cell types of melanocyte (n = 3) and lymphocyte origin (n = 8). The corresponding patterns of DNase I HS in these cell types suggest that *IRF4* expression in lymphocytes and melanocytes is directed by distinct sets of regulatory elements (Figure 2B). In addition, the position orthologous to rs12203592 in the mouse genome directly overlaps a melanocyte enhancer (Gorkin et al., 2012) because it is occupied by p300 and marked by H3K4me1 in the melanocyte line melan-Ink4a-Arf (Sviderskaya et al., 2002) (Figure 2C).

To confirm that the intronic sequence containing rs12203592 acts as a melanocyte-specific enhancer, we subcloned a 450 bp fragment containing the rs12203592-C allele upstream of a minimal promoter that drives luciferase expression and assayed its activity in both the mouse melan-Ink4a-Arf and

human SK-MEL-28 cell lines. The fragment showed strong enhancer activity, directing >35-fold-higher luciferase expression than the minimal promoter alone in melan-Ink4a-Arf, and >200-fold in SK-MEL-28 (Figures 3A and 3B). We next asked whether the genotype at rs12203592 affects enhancer activity. In both melan-Ink4a-Arf and SK-MEL-28, the presence of the rs12203592-T allele significantly reduced the enhancer activity of the fragment (p < 0.008) (Figures 3A and 3B).

We further assayed the sequence containing rs12203592 in vivo using transgenic zebrafish. We engineered a vector containing the entire human *IRF4* intron 4 sequence upstream of a minimal promoter and the gene encoding GFP. We created two versions of the vector, with either the T or C allele at rs12203592; as above, the remainder of the sequence was identical in both constructs. We injected these constructs, or a negative control construct lacking any human genomic sequence, into zebrafish embryos at the two-cell stage, incubated them 48 hr postfertilization, and scored them for the presence of GFP-positive cells. In embryos injected with the negative control construct, we did not detect GFP-positive melanocytes (0 out of 40 embryos) (Figure 3C). In embryos injected with the *IRF4* intron 4 reporter construct containing the ancestral rs12203592-C allele, we detected GFP-positive melanocytes in about 20% of embryos (8 out of 40 embryos; 17 melanocytes total from the 8 embryos), consistent with the mosaicism expected in transient transgenic embryos (Figures 3C and 3D). By contrast, in embryos injected with the reporter containing rs12203592-T, only 9% of the embryos had detectable GFP-positive melanocytes (4 out of 44 embryos; only six melanocytes total). The difference in the total number of melanocytes is statistically significant (p = 0.0023, unpaired t test). We did not detect expression in other tissues, beyond transient, scattered expression, which was seen in all three constructs and has been reported by others (Bessa et al., 2009). We conclude that *IRF4* intron 4 contains a melanocyte enhancer, likely directing *IRF4* expression in these cells, and that the rs12203592-T allele reduces the activity of this enhancer.

MITF Activates IRF4 Gene Expression

Chromatin immunoprecipitation sequencing (ChIP-seq) and gene expression studies have suggested that MITF may be involved in regulating *IRF4* gene expression (Hoek et al., 2008, Strub et al., 2011). We analyzed *Irf4* expression in mice lacking *Mitf* and focused on the heart because cells that normally express *Mitf* are still present in the heart of *Mitf*[mi-vga9] homozygous mutants, whereas melanocytes are absent resulting in white coat color (Hodgkinson et al., 1993). *Irf4* gene expression was dramatically reduced in *Mitf* mutants compared to wild-type controls (Figure 4A), consistent with the possibility that *Irf4* is regulated by MITF. To determine whether *IRF4* is a target of MITF in the melanocyte lineage, we used shRNA to knock down *MITF* and *IRF4* mRNAs in human 501mel melanoma cells, which normally express these genes. Transfecting these cells with shRNA directed against *MITF* reduced *MITF* mRNA expression to 45% (Figure 4B) and MITF protein levels to 41% (Figures 4C and S2) of those seen in untreated cells or cells treated with a scrambled control shRNA. In cells treated with shMITF, the expression of *IRF4* mRNA and protein was dramatically reduced (to 50% and 25%, respectively). shRNA against *IRF4*
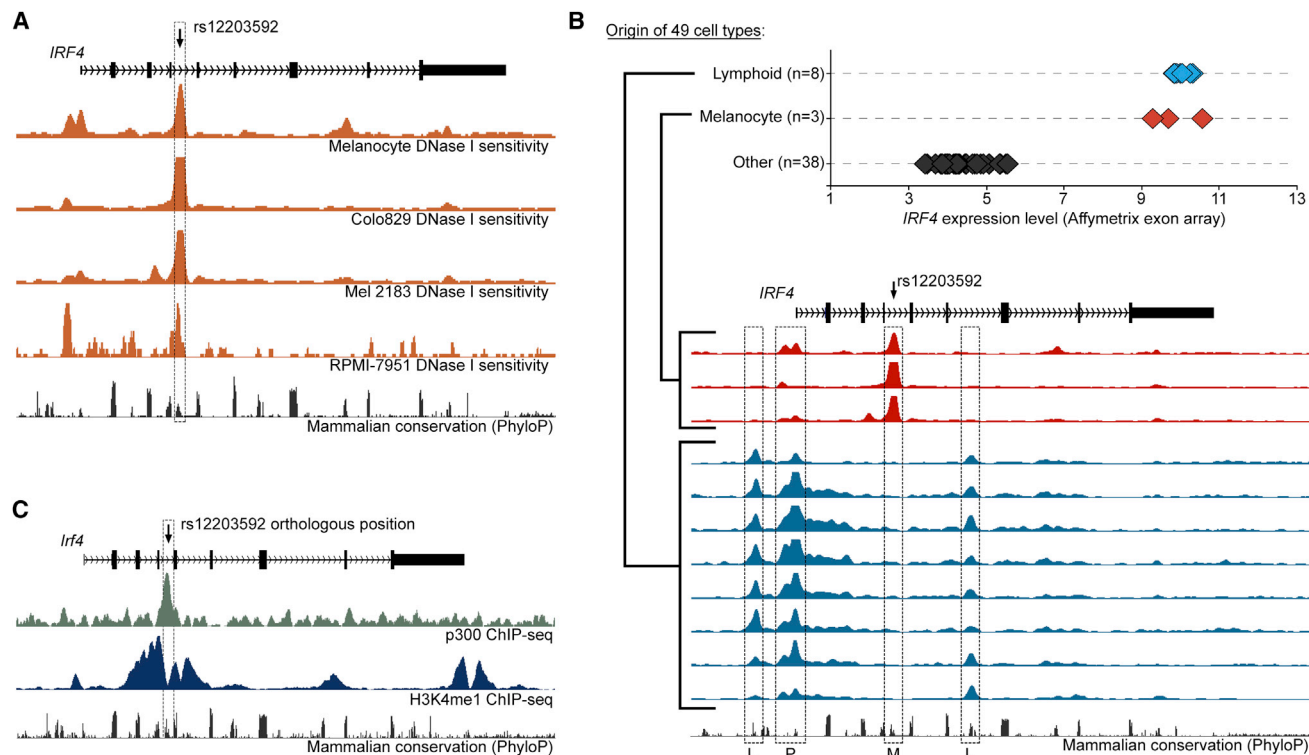
Figure 2. rs12203592 Disrupts a Conserved Melanocyte Enhancer at *IRF4*

(A) UCSC genome browser view shows 25 kb region around *IRF4* (hg19 coordinates chr6:388,750–413,750). DNase-seq signal (orange) in human epidermal melanocytes, Colo829, Mel 2183, and RPMI-7951 was generated by ENCODE.

(B) Top view is a graph showing *IRF4* expression level as measured by ENCODE in 49 cell types (Duke Affymetrix exon arrays). Bottom is the UCSC genome browser view of 25 kb region around *IRF4* (hg19 coordinates chr6:388,750–413,750) in corresponding cell lines. Melanocyte-specific DNase HS peak overlapping rs12203592 is labeled "M," lymphoid-specific DNase HS peaks are labeled "L," and DNase HS overlapping the IRF4 promoter is labeled "P." Cell types shown are (top to bottom) human epidermal melanocytes, Colo829, Mel 2183, GM12878, GM12891, GM12892, GM19238, GM19238, GM19240, GM18507, and CLL.

(C) UCSC genome browser view shows 25 kb region around *Irf4* (mm9 coordinates chr13:30,838,000–30,863,000). The ChIP-seq signals for EP300 (green) and H3K4me1 (blue) in the mouse melanocyte line melan-Ink4a-Arf are from Gorkin et al. (2012).

resulted in reduction in *IRF4* mRNA and protein expression to 40% and 20% of control levels, respectively, whereas *MITF* expression was unaffected (Figures 4C and S2). The expression of *DCT* (encoding Dopachrome tautomerase) and TYR, two known MITF target genes (Yasumoto et al., 1994, 2002), was also reduced upon shMITF treatment, whereas shIRF4 treatment only affected the expression of *TYR* (Figure 4B). TYR protein expression was reduced upon treatment with shMITF, shIRF4, and shAP2A, as well as with all shRNAs together (Figure S2). Knocking down TFAP2A did not affect MITF expression. We also tested this relationship in an overexpression assay. Untransfected 501mel melanoma cells express *MITF* but low levels of both *TYR* and *IRF4* mRNAs, whereas HEK293T cells do not express *MITF* endogenously and also lack expression of *TYR* and *IRF4*. In both cell lines, overexpression of mouse *Mitf* (*mMitf*) strongly induced the levels of *TYR* and *IRF4* (Figure 4D). Analysis of the expression of MITF and IRF4 in 22 melanoma cell lines revealed a positive correlation between MITF and IRF4 (p = 0.0003, Pearson correlation coefficient, Figure S3A). These results indicate that MITF directly or indirectly regulates *IRF4* expression. They also suggest that *TYR* expression depends on IRF4.

rs12203592 Alters the Function of a Melanocyte Enhancer in IRF4 through Disruption of a TFAP2A-Binding Site

The rs12203592 polymorphism is located 66 bp from three sites recently shown to be occupied by MITF in ChIP-seq studies (Figure 5A) (Strub et al., 2011). ChIP performed in 501mel and MITF-transfected 293T cells showed that MITF binds to intron 4 of *IRF4* (Figure 5B). Interestingly, the rs12203592-T polymorphism occurs in a predicted binding site (GGCAAA) for TFAP2A (Do et al., 2010), which was recently shown to be involved in melanocyte differentiation in zebrafish (Van Otterloo et al., 2010). To determine if TFAP2A can bind this sequence, we performed anti-TFAP2A ChIP in 501mel cells (homozygous for rs12203592-C) and showed that TFAP2A binds to the intron 4 element in the *IRF4* gene (Figure 5C). Furthermore, gel shift assays showed that TFAP2A binds oligos carrying the TFAP2A motif from *IRF4* intron 4 only when the oligos harbor the rs12203592-C allele. It did not bind the rs12203592-T variant, nor when the binding site was altered completely (Figure 5D). Addition of antibody resulted in a supershift only in the presence of the wild-type oligo. Binding of MITF to the neighboring MITF sites was not affected by the SNP (Figure S3B). These results show that TFAP2A binds
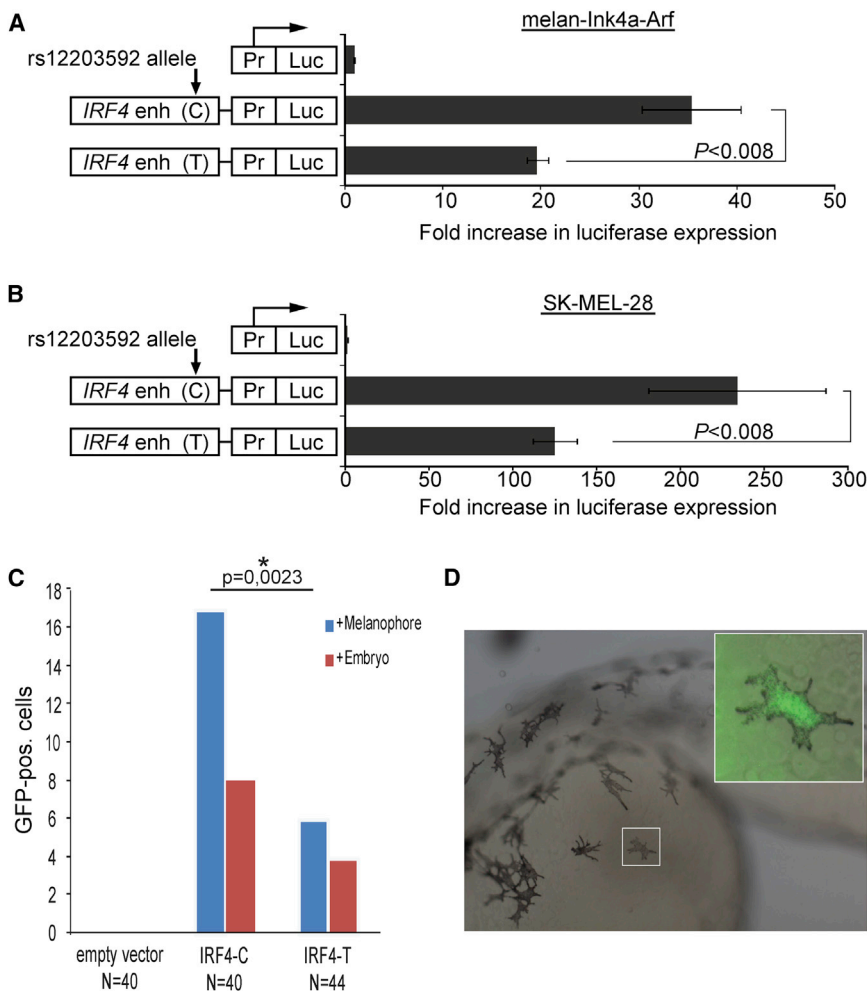
Figure 3. Intron 4 of IRF4 Drives Expression in Melanocytes

(A and B) Results of the luciferase assays in melan-Ink4a-Arf and SK-MEL-28 cells are shown. Pr, promoter; Luc, luciferase reporter gene; *IRF4* enh, 450 bp fragment from the fourth intron of IRF4 containing rs12203592. "*IRF4* enh" fragments are identical except for the base at position rs12203592 as indicated ("C" is the ancestral allele; "T" is the derived allele). The x axis shows fold luciferase activity relative to the minimal promoter alone, which is normalized to one (1). p values are calculated by Kolmogorov-Smirnov test. The result is also highly significant by other nonparametric tests (p = 0.007937 by Wilcoxon rank sum test for both melan-Ink4a-Arf and SK-MEL-28) and by the standard parametric t test (p = 0.001686 for melan-Ink4a-Arf; p = 0.01032 for SK-MEL-28). Each bar represents the average of five biological replicates per reporter construct. Error bars represent SD.

(C) Reporter constructs contain either the rs12203592-C version of the IRF4 intron 4 element (IRF4) or the rs12203592-T version (IRF4snp) upstream of the GFP reporter after injection into zebrafish. The numbers represent the number of melanophores (blue) and embryos (red) seen to be positive (pos.) for GFP. The difference between the melanophores containing the wild-type allele of intron 4 of IRF4 compared to the mutant allele (rs12203592) is statistically significant (*p = 0.0023, unpaired t test).

(D) Image shows a GFP-positive zebrafish embryo with a GFP-positive melanophore in the inset.

the ancestral sequence (rs12203592-C) in intron 4 of *IRF4* but fails to bind to the rs12203592-T variant.

To determine if TFAP2A plays a role in the activity of the *IRF4* enhancer, we performed reporter assays using intron 4 of *IRF4* as regulatory element. 501mel melanoma cells were cotransfected with the reporter constructs and with plasmids encoding MITF, TFAP2A, or both. Neither MITF nor TFAP2A alone elevated expression from this element above basal levels (Figure 5E). However, when both MITF and TFAP2A were expressed simultaneously, transcription was increased 6-fold (Figure 5E), suggesting that the proteins have cooperative effects on the activity of this enhancer. This is further supported by the shRNA experiments that showed that knocking down TFAP2A reduces IRF4 expression (Figures 4B and 4C). Removing all three MITF-binding sites together (intron 4-3xE) led to background-level expression (Figure 5E). Importantly, when the rs12203592-T variant was introduced into the reporter construct (intron 4-rs), the cooperation seen between MITF and TFAP2A was reduced to levels close to background (Figure 5E). This suggests that MITF requires TFAP2A in order to induce expression from the intron 4 element and that the rs12203592-T polymorphism abolishes the MITF-mediated activation.

To determine whether the rs12203592-T variant affects *IRF4* expression in melanocytes, RNA and protein were isolated from melanocytes differentiated from human neonatal foreskin melanoblasts from Australian subjects (Cook et al., 2009). RT-qPCR showed a significantly lower (p < 0.01, unpaired t test) expression of *IRF4* mRNA in cells homozygous for the rs12203592-T polymorphism than in cells homozygous for the ancestral C (Figures 5F and S3C). IRF4 protein levels in cells from T/T individuals were considerably reduced compared to that of cells from C/C individuals (Figure 5G).

To determine whether the rs12203592 polymorphism affects expression of the transcript on the chromosome on which the variation resides, we used an allele discrimination assay. We used RT-qPCR on human primary melanocytes heterozygous for the *IRF4* 3′ UTR SNP rs872071-A/G. The rs872071-G allele resides 15 kb distal to rs12203592 and is present on the haplotype on which the rs12203592-T allele arose, thus allowing for comparison of IRF4 allele-specific expression levels, controlling for potential transfactor variations that may contribute to expression level differences between individuals. We observed no difference in IRF4 allelic expression in rs12203592-C/C homozygous melanocytes, as represented by the rs872071-A/G allele ratio (Figure S4). In contrast, cells heterozygous for the rs12203592 C/T alleles exhibit a significantly higher
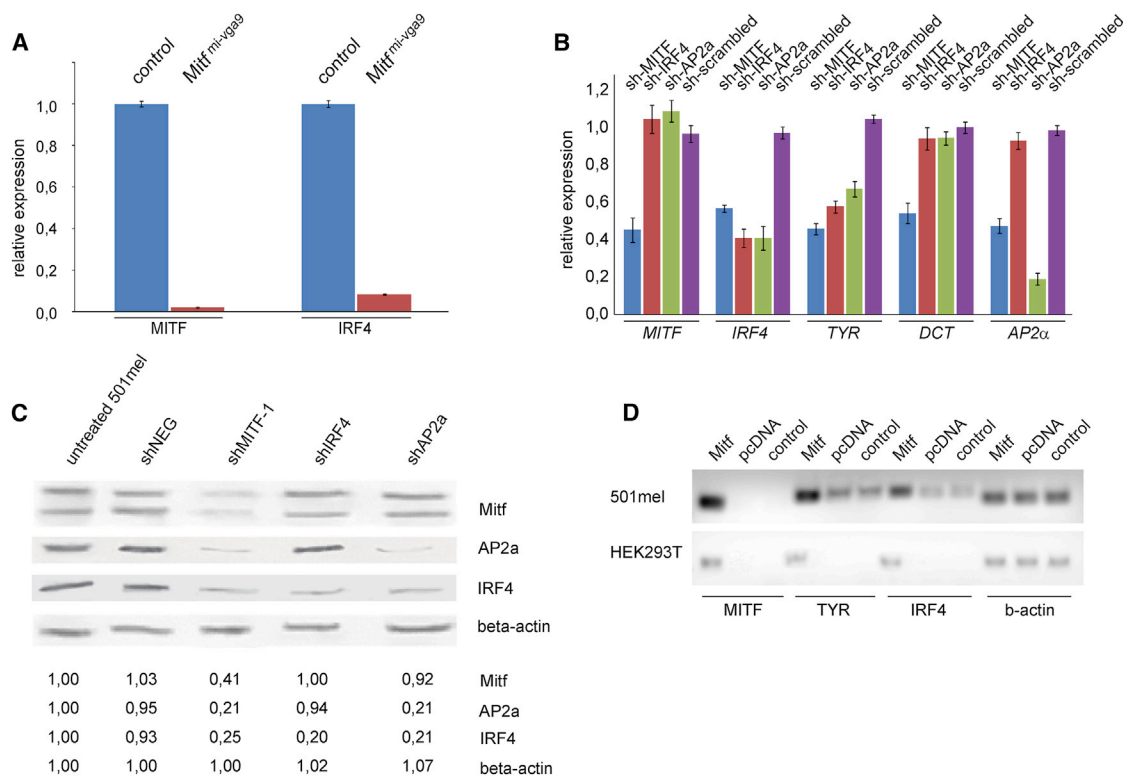
Figure 4. MITF Regulates Expression of IRF4

(A) The expression of the *Mitf* and *Irf4* mRNAs is reduced in hearts from *Mitf*[mi-vga9] mutant mice, as determined by qPCR analysis. Data are represented as mean ± SEM.

(B) Expression of the *MITF*, *IRF4*, *TFAP2a*, *DCT*, and *TYR* genes upon treatment with shRNA against *MITF*, *IRF4*, *TFAP2A*, and negative control in 501mel melanoma cells as determined by qPCR analysis is shown. Data are represented as mean ± SEM.

(C) Western blot shows expression of the MITF, TFAP2A, IRF4, and β-actin proteins in 501mel cells after shRNA treatment. Intensity quantification is relative to actin-loading control. See also Figure S2.

(D) Overexpression of the mouse Mitf (mMitf) cDNA (expressed from pcDNA3.1) in 501mel melanoma cells and in HEK293T embryonic kidney cells affects expression of the TYR and IRF4 mRNAs as assayed by RT-qPCR, whereas β-actin expression is unchanged. mMitf was detected with species-specific primers that only recognize the mouse *Mitf* gene. See also Figure S3.

See also Figure S2 and Table S3.

rs872071-A/G allele ratio (p < 0.01), consistent with reduced transcription occurring from IRF4 rs872071-G alleles in phase with rs12203592-T.

Several alternate products are made from the IRF4 gene, including transcripts that initiate in intron 4 (Figure S5A), leading to an mRNA containing internal ATGs (lacking Kozak consensus sequences) and the tentative production of a truncated protein lacking the N-terminal DNA-binding domain. To determine the major product in the melanocyte, RT-qPCR analysis with transcript-specific primers was performed on RNA isolated from foreskin melanoblasts from Australian subjects. This showed that the major *IRF4* product in melanocytes is the full-length transcript; the smaller alternative product was barely detectable (Figure S5B). Similarly, in mouse melanoblasts isolated from E15.5 and E17.5 embryos and melanocytes from P1 and P7 neonatal pups (Debbache et al., 2012), the full-length *Irf4* transcript was the only transcript detected at all developmental stages (Figure S6). We therefore conclude that the regulatory element in intron 4 affects the production of the full-length *IRF4* transcript.

IRF4 and MITF Cooperatively Regulate TYR Expression

To determine how IRF4 levels might affect pigmentation, we searched the promoter sequences of several pigmentation genes for the IRF4-binding motif. Several potential IRF4-binding motifs were found in the *TYR* promoter, on either side of known MITF-binding sites previously shown to be essential for gene activation (Figure 6A) (Yasumoto et al., 1994; Bertolotto et al., 1996). To investigate the relationship between IRF4 and the expression of several pigmentation genes, two human melanocyte strains with high expression of *IRF4* were independently transfected with siRNA targeting *IRF4* for 48 hr, followed by total RNA extraction and RT-qPCR. These results combined show that expression of *IRF4* was reduced on average to 40% of cells treated with negative control siRNA (Figure 6B). Interestingly, the expression of *TYR* was reduced to 50% compared to untreated control, whereas the expression of the pigmentation genes *SLC45A2* (*MATP*), *SLC24A5*, and *DCT* was largely unaffected (Figure 6B). Similarly, knockdown of *IRF4* affected expression of *TYR* but not of *DCT* in 501mel melanoma cells (Figures 2B and 2C). This suggests that IRF4 is involved in regulating *TYR* expression but
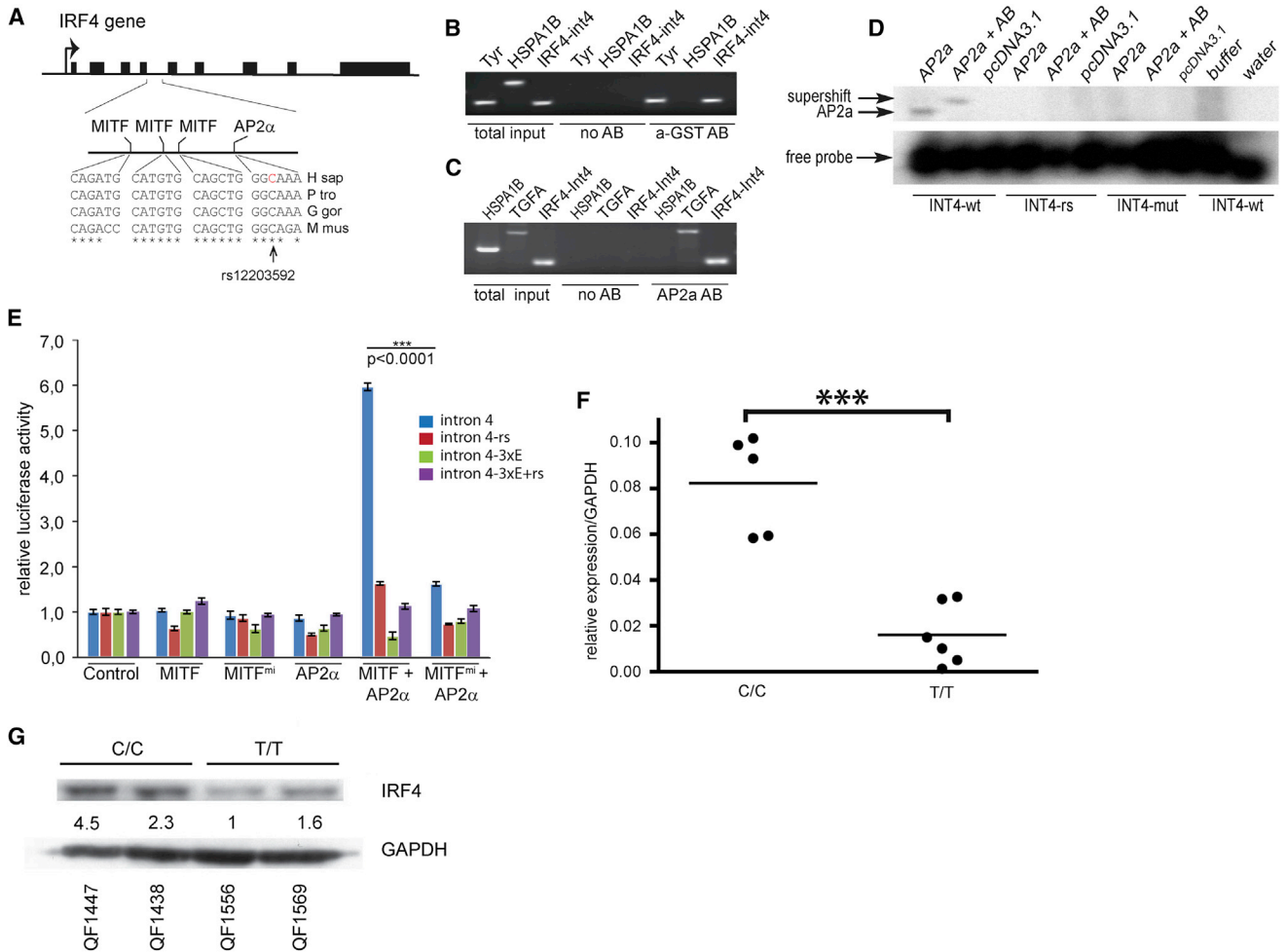
Figure 5. MITF and TFAP2A Affect *IRF4* Expression by Binding Regulatory Elements in Intron 4

(A) Schematic view presents the IRF4 gene. The sequence shows a comparison of MITF- and TFAP2A-binding sites in intron 4 of IRF4 among humans (H sap [*Homo sapiens*]), gorilla (G gor [*Gorilla gorilla*]), chimpanzee (P tro [*Pan troglodytes*]), and mouse (M mus [*Mus musculus*]). The location of the rs12203592 polymorphism is indicated.

(B) ChIP analysis of a GST-tagged MITF protein (a-GST AB) was performed in 501mel cells. Primers specific for HSPA1B (negative control), TYR (positive control), and intron 4 of IRF4 are indicated. The GST-tagged Mitf only precipitated TYR and IRF4 sequences.

(C) ChIP analysis of TFAP2A in 501mel cells is presented. PCR products specific for TGFA (positive control), HSPA1B (negative control), and intron 4 of IRF4 are shown. TFAP2A only precipitated TGFA and INT4 sequences.

(D) Gel shift analysis shows the binding of TFAP2A to the ancestral sequence (INT4-WT) in intron 4 of IRF4 (coordinates chr6: 396309–396333 in build GRCh37.p5 of the human genome), but not to the rs12203592-T sequence (INT4-RS) or to a completely mutated sequence (INT4-mut).

(E) Luciferase reporter assays were performed in 501mel melanoma cells using intron 4 of IRF4 as a reporter. The ancestral IRF4 intron 4 sequence (intron 4, blue), the rs12203592-T polymorphic sequence (intron 4-rs), a sequence where all MITF-binding sites were mutated (intron 4-3xE), and a sequence containing mutated MITF sites and the rs12203592-T polymorphism (intron 4-3xE+rs) were tested. The luciferase reporters were cotransfected with the wild-type MITF and TFAP2A proteins and with a dominant-negative version of MITF (MITF^{mi}). Statistical analysis was done using unpaired t test. Data are represented as mean ± SEM.

(F) Cells homozygous for the ancestral allele (CC) express significantly higher levels of IRF4 than cells homozygous for the rs12203592-T polymorphism (TT). Fold expression data are represented relative to pooled mean TT expression ± SEM. Statistical analysis was performed using ANOVA (***p < 0.001).

(G) A western blot shows expression of the IRF4 protein in melanocytes from CC and TT individuals. Intensity quantification is relative to GAPDH-loading control. Note the decrease of basal IRF4 protein in the TT melanocyte cell lines.

See also Figures S3, S4, and S5.

does not affect expression of SLC45A2 or SLC24A5. The expression of IRF4 correlates with the expression of TYR in 22 melanoma cell lines (p = 0.0007, Pearson correlation coefficient, Figure S3A).

Cotransfection assays in HEK293T cells (which do not express MITF) showed that, whereas MITF can activate expression of the TYR gene, the IRF4 protein is unable to do so on its own (Figure 6C). However, in the presence of both MITF and IRF4, cooperative effects were observed with increasing amounts of IRF4 (Figure 6C). A mutant MITF protein with defective DNA-binding ability resulted in less activation and reduced cooperativity
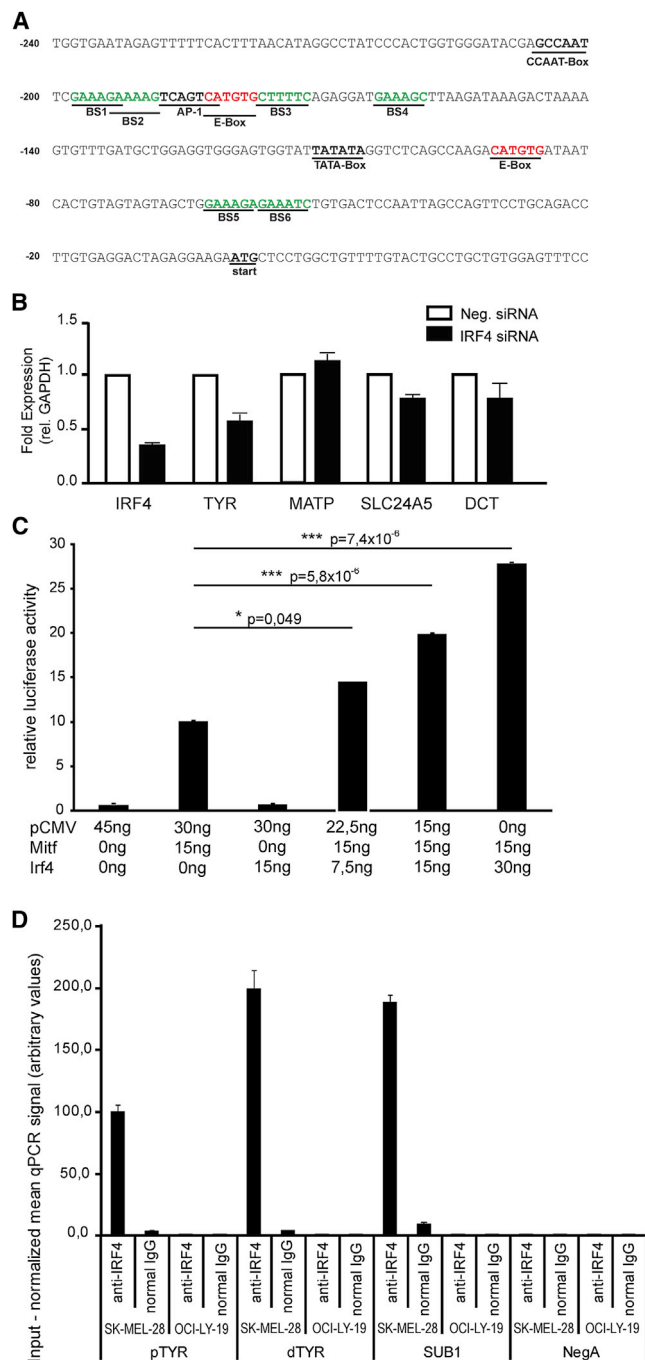
A

```
-240  TGGTGAATAGAGTTTTTCACTTTAACATAGGCCTATCCCACTGGTGGGATACGAGCCAAT
                                                              CCAAT-Box

-200  TCCGAAAGAAAAGTCAGTCATGTGCTTTTCAGAGGATGAAAGCTTAAGATAAAGACTAAAA
         BS1      BS2  AP-1 E-Box  BS3        BS4

-140  GTGTTTGATGCTGGAGGTGGGAGTGGTATTATATAGGTCTCAGCCAAGACATGTGATAAT
                                     TATA-Box               E-Box

-80   CACTGTAGTAGTAGTCTGGAAAGAGAAATCTGTGACTCCAATTAGCCAGTTCCTGCAGACC
                         BS5    BS6

-20   TTGTGAGGACTAGAGGAAGAATGCTCCTGGCTGTTTTGTACTGCCTGCTGTGGAGTTTCC
                            start
```

B

C

pCMV	45ng	30ng	30ng	22,5ng	15ng	0ng
Mitf	0ng	15ng	0ng	15ng	15ng	15ng
Irf4	0ng	0ng	15ng	7,5ng	15ng	30ng

D

Figure 6. MITF and IRF4 Regulate Expression of TYR

(A) Schematic view of the human TYR promoter shows the sequence of the MITF and potential IRF4-binding sites.

(B) Expression of IRF4, TYR, MATP, SLC24A5, and DCT was determined by qPCR, after treatment with siIRF4 or control siRNAs. Fold expression of each gene is represented relative to cells transfected with negative siRNA (arbitrarily set to one [1]). Data represent the mean ± range of two experiments using two primary melanocytes (QF1438 and QF1424).

(C) Luciferase reporter assay used wild-type TYR promoter as reporter construct and cotransfected with constructs expressing the MITF and IRF4 proteins. Statistical analysis was performed using the t test; data are represented as average with the SD.

(Figure S7A). Similar effects were observed when the E box was mutated (Figure S7B), confirming that the cooperativity is mediated through MITF. Quantitative ChIP experiments in Skmel28 (Figure 6D) and Skmel5 (Figure S7C) melanoma cells showed that IRF4 binds the proximal TYR promoter as well as a more distant region.

Irf4 Affects Pigmentation in Mice

No pigment phenotypes were reported in mice with a targeted deletion of *Irf4* (Mittrücker et al., 1997). We analyzed the expression of *Mitf*, *Tfap2a*, *Irf4*, and *Tyr* in melanoblasts isolated from E15.5, E17.5, P2, and P7 mouse embryos (Debbache et al., 2012). *Mitf* and *Tfap2a* are both expressed at the embryonic and postnatal stages, whereas *Irf4* is detected at a low level at the two postnatal stages (Figure 7A). *Tyr* is expressed at all stages, but the level of expression increases at the postnatal stages and is 10-fold higher at P7 than at the earlier stages (Figure 7A). This expression pattern is consistent with our data that MITF and TFAP2A regulate *IRF4* expression, and MITF and IRF4 regulate *TYR* expression. Because early stages of development do not express *Irf4*, this also suggests that IRF4 is mostly involved in melanocyte differentiation. To determine if *Irf4* plays a role in mouse melanocytes, mice carrying an *Irf4-flox* cassette (*Irf4 f*) (Klein et al., 2006) were crossed to mice expressing the Cre-recombinase under control of the *Tyr* promoter (*Tyr::Cre*) (Delmas et al., 2003). This led to the lack of *Irf4* expression in *Tyr*-expressing cells, but no apparent pigmentation phenotype, similar to the *Irf4* null mice (Mittrücker et al., 1997). In order to test the relationship between *Mitf* and *Irf4* in the mouse, we crossed the *Tyr::Cre/⁰*; *Irf4* f/f mice to mice carrying the dominant-negative allele *Mitf^{Mi-White}* (*Mitf^{Mi-Wh}*). Mice heterozygous for this allele (*Mitf^{Mi-Wh}*/+) have a partial deficiency of *Mitf* and exhibit a stable, gray coat color with white belly spot. Interestingly, the *Tyr::Cre/⁰*; *Irf4* f/f; *Mitf^{Mi-Wh}*/+ mice showed distinctly lighter coat color than seen in *Mitf^{Mi-Wh}*/+ mice, which are negative for the *Tyr-Cre* transgene (*Irf4* f/f; *Mitf^{Mi-Wh}*/+) (Figure 7B). This confirms that IRF4 plays a role in mouse pigmentation, like in humans, and that the effects of *Irf4* in the mouse also depend on *Mitf*. In humans, the *IRF4* variant rs12203592-T is linked to brown hair color (Sulem et al., 2007) (Table S1). Thus, reduced *IRF4* expression alone is sufficient for lighter hair color in humans, whereas in mice, MITF function also needs to be reduced to see effects on pigmentation. This suggests that there may be species-specific differences between mice and humans in terms of the requirement for *MITF* and *IRF4* in melanocytes of the hair.

(D) IRF4 binds the TYR locus. ChIP real-time PCR (ChIP-qPCR) analysis shows IRF4 binding at sites proximal (around transcription start site; pTYR) and more distal (∼1,800 bp upstream of transcription start site; dTYR) to TYR-coding region and other control loci in SK-MEL-28 melanoma cell line and in negative control OCI-Ly-19, a germinal center type B cell lymphoma (GCB-DLBCL) cell line previously shown to lack IRF4 expression (Yang et al., 2012). Primer pair amplifying a region upstream of the SUB1 locus was used as a positive control because this region showed strong IRF4 binding both in multiple myeloma (Shaffer et al., 2008) and activated B cell-type B cell lymphoma (Yang et al., 2012) cell lines. NegA is a region on chromosome 7, used as a negative control for IRF4 binding, due to the lack of observable IRF4 binding in previous studies with multiple myeloma and ABC-DLBCL cell lines. Error bars depict SEM. See also Figure S7.
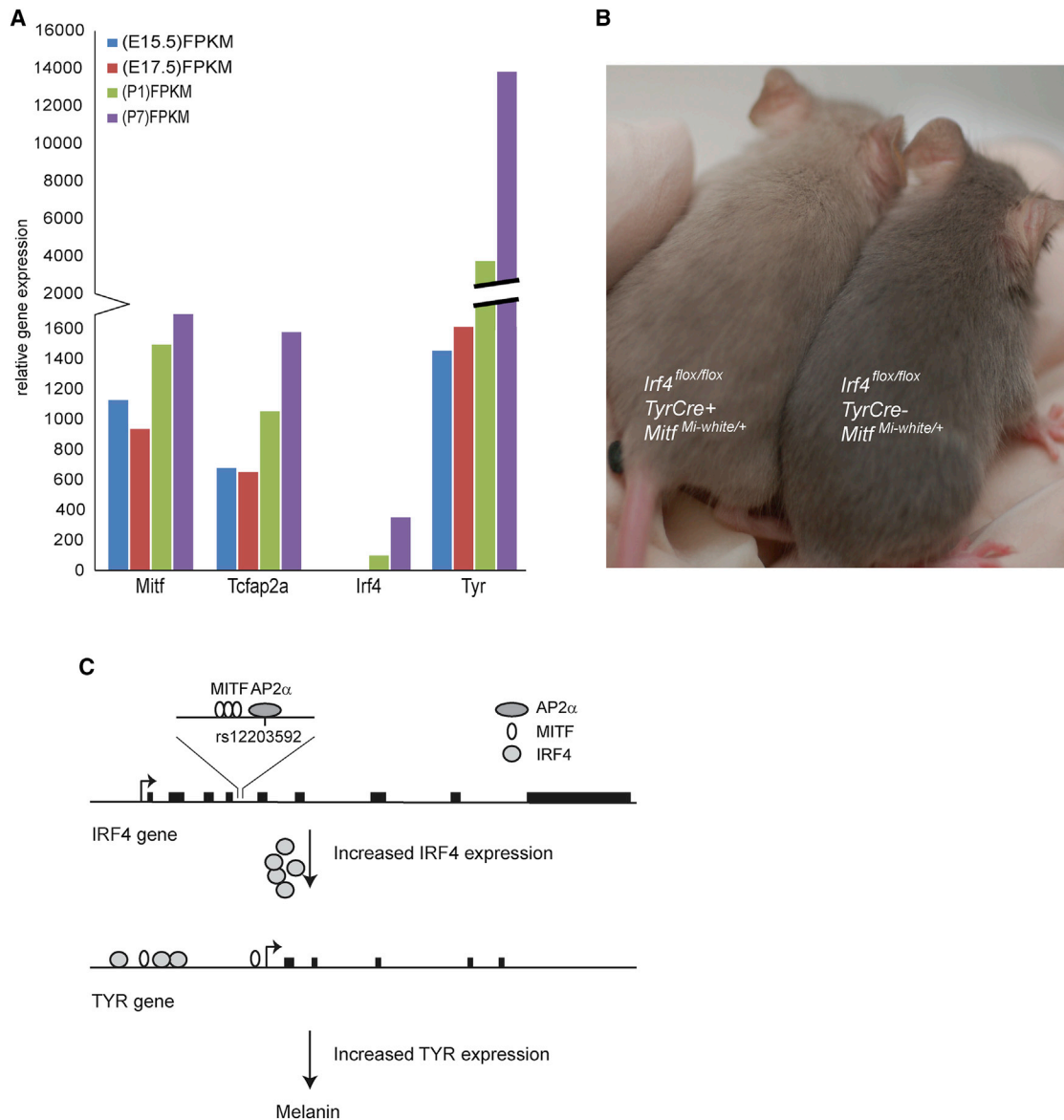
Figure 7. IRF4 Is Involved in Regulating Pigmentation in Mice

(A) The expression plot shows the presence and clear differences in the expression of *Mitf*, *Tcfap2a*, *Irf4*, and *Tyr* at different developmental time points, E15.5, E17.5, P1, and P7, measured in FPKM. Indicated expression levels of a transcript are proportional to the number of reads sequenced from that transcript after normalizing for respective transcript's length. RNA from E15.5 embryos is shown in blue bars, E17.5 embryos in red, P1 pups in green, and P7 in violet.

(B) Mice where *Irf4* has been conditionally knocked out in heterozygotes for the semidominant *Mitf^{Mi-Wh}* mutation are presented. *Mitf^{Mi-Wh}* heterozygotes that are simultaneously lacking *Irf4* show lighter coat color (left) than mice that are wild-type for *Irf4* (right).

(C) Model depicts the relationship among MITF, TFAP2A, and IRF4 in melanocytes and their effects on the expression of *TYR*. MITF and TFAP2A bind together to the intron 4 element in the *IRF4* gene and regulate the expression of *IRF4* from the upstream promoter. MITF and IRF4 then bind to and activate expression of TYR, leading to normal pigmentation. The rs12203592-T polymorphism leads to reduced IRF4 activation and thus reduced TYR expression, which consequently leads to sun sensitivity and blue eyes.

DISCUSSION

GWASs have yielded a long list of sequence variants correlated with a wide range of human phenotypes. Most of these variants are located in noncoding regions of the genome and do not alter the amino acid sequence of proteins or have known regulatory functions. Two steps are essential in order to characterize the biology behind such associations. First, the association signals must be fine mapped using full sequence data. Second, the biological processes involved must be unraveled experimentally. In this study, we have taken both steps to shed light on the functional consequences of the IRF4-noncoding variant rs12203592 on pigmentation.

Our work proposes a model describing how IRF4 acts in cooperation with MITF to influence pigmentation phenotypes (Figure 7C). In melanocytes, MITF and TFAP2A cooperatively activate *IRF4* expression through an enhancer element in intron 4. In turn, IRF4 and MITF cooperate to activate transcription of *TYR*. The rs12203592-T allele reduces binding of TFAP2A to its cognate site in intron 4 of *IRF4*, thereby suppressing the induction of IRF4 expression and, as a consequence, impairing the cooperative induction of *TYR*. This leads to the pigmentation phenotypes associated with the rs12203592-T allele. The effects of this variant must be cell-type specific because Do et al. (2010) have shown that it leads to increased *IRF4* promoter activity in Burkitt lymphoma B cells (Raji), HEK293T human embryonic kidney cells, and the NCI-H295R human adrenal cells. Consistent with our results, they showed that TFAP2A binds with higher affinity to the C allele than to the T allele (Do et al., 2010). Thus, the cell-type-specific effects must be mediated by transcription factors other than TFAP2A.

In humans, genetic variants in different genes have particular effects on pigmentation traits. For example, *MC1R* and *ASIP* variants have major effects on freckling, sun sensitivity, and red hair, whereas they have relatively little effect on eye color (Sulem et al., 2007, 2008). The rs12203592-T variant of *IRF4* also has major effects on freckling and sun sensitivity but is associated with brown (over blond) hair and has a notable effect on eye color (Table S1). The results presented here suggest that *IRF4*, like *MC1R* and *ASIP*, exerts its major effects on pigmentation traits through the master regulator MITF. However, some divergence in pathways must occur in order to account for the differences in effects on pigmentation traits. We have shown that *TYR* is a target of MITF-IRF4 cooperative activation. However, *TYRP1* and *DCT* are targets of MITF activation that do not appear to be responsive to IRF4. Clearly, the MITF-IRF4 cooperation only occurs in a subset of MITF-responsive genes. A broader search of MITF target genes for potential IRF4 responsiveness might shed light on the pattern of pigmentation traits affected by *IRF4*.

Freckles represent clusters of concentrated melanin in the skin, without an increase in melanocyte numbers. Freckles are either light brown or red but usually become darker and more visible upon exposure to sunlight. GWASs have shown that variations in MC1R, ASIP, TYR, and BNC2, in addition to IRF4, are associated with freckles (Sulem et al., 2007, 2008; Eriksson et al., 2010). Mutations in the *MC1R* gene have been highly associated with the formation of freckles (Bastiaens et al., 2001). In addition to regulating TYR and IRF4 gene expression, MITF is also known to regulate expression of *MC1R* (Adachi et al., 2000; Smith et al., 2001; Aoki and Moro, 2002). This suggests the possibility that, like with TYR, IRF4 cooperates with MITF in regulating expression of *MC1R* in melanocytes. Indeed, ChIP-seq studies show that MITF binds MC1R regulatory sequences (Strub et al., 2011).

The effects of both TFAP2A and IRF4 are mediated through MITF, which is needed both for the TFAP2A-mediated activation of *IRF4* and the IRF4-mediated activation of *TYR*. ChIP-seq studies suggest that MITF regulates *TFAP2A* as well as *IRF4* (Strub et al., 2011). The cooperative effects of MITF and IRF4 suggest that these proteins might physically interact on the *TYR* promoter. TFAP2A is expressed in a variety of both mesen-

chymal and epithelial cell types. It is not known how MITF and TFAP2A interact in the regulation of IRF4. Because the binding sites are physically close to each other, it is possible that both proteins are part of a larger complex formed at the site. Together, these data suggest that transcription factors that are expressed in multiple cell types can form cell-type-specific complexes to govern differentiation.

Several studies have shown that polymorphisms identified as susceptibility loci in GWASs affect the binding of particular transcription factors, in some cases, leading to differences in expression of a nearby target gene (Meyer et al., 2008; Rahimov et al., 2008; Pomerantz et al., 2009; Schödel et al., 2012; Tuupanen et al., 2009). However, the effects of the gene expression differences have not been characterized, and more importantly, the biological effects on the phenotype are only implied in these studies. A recent example is provided by the rs12913832 polymorphism in intron 86 of the *HERC2* gene, which shows a strong association with human eye color and pigmentation. Because the pigment gene *OCA2* is located 21 kb downstream of *HERC2*, it has been speculated that this polymorphism is located in a distal regulatory element of OCA2 and may affect binding of the transcription factors HLTF, LEF1, and MITF (Visser et al., 2012). This results in the formation of a loop between the HERC2 enhancer and the OCA2 promoter, thus affecting OCA2 expression. The rs12913832-C allele prevents HLTF from binding to the element, thus reducing loop formation. The regulation of OCA2 expression may be more complex, however, with separate regulatory elements responsible for eye and skin color (Beleza et al., 2013). Here, we have gone one step further and shown the effects of transcription factor binding on gene expression and how this gene expression difference leads to effects on human pigmentation.

EXPERIMENTAL PROCEDURES

Whole-Genome Sequence Analysis
Icelandic Population Samples and Phenotypic Information
Icelandic adults were recruited as cases, family members, or controls through a series of cardiovascular, oncology, neurology, and metabolic studies conducted by deCODE Genetics. Blood samples were taken for DNA isolation upon recruitment. The studies were approved by the National Bioethics Committee of Iceland and the Icelandic Data Protection Commission. Personal identifiers associated with phenotypic information, blood samples, and genotypes were encrypted using a third-party encryption system. Each participant completed a questionnaire that included questions about natural eye color categories (blue/gray, green, or black/brown), natural hair color categories (red/reddish, blond, dark blond/light brown, or brown/black), and the presence of freckles at any time. Sun sensitivity was assessed using the Fitzpatrick skin-type score (Fitzpatrick, 1988), where the lowest score (I) represents very fair skin that is sensitive to UVR, and the highest score represented in native Icelanders (IV) represents dark skin that tans rather than burns in reaction to UVR exposure. Individuals scoring I or II were classified as being sensitive to sun, whereas individuals scoring III and IV were classified as not being sensitive to sun.

SNP ChIP Genotyping
The Icelandic chip-typed samples were genotyped with Illumina Human Hap300, HumanHap CNV370, HumanHap 610, 1 M, Omni1-Quad, Omni 2.5, or Omni Express bead chips at deCODE Genetics. SNPs were excluded if they had (1) yield less than 95%, (2) minor allele frequency less than 1% in the population, (3) significant deviation from Hardy-Weinberg equilibrium in the controls (p < 0.001), (4) if they produced an excessive inheritance error

rate (over 0.001), and (5) if there was a substantial difference in allele frequency between the chip types (SNPs from a single-chip platform were excluded if that resolved all differences but otherwise from all chip platforms). All samples with a call rate below 97% were excluded from the analysis. For the Human-Hap series of chips, 308,840 SNPs were used for long-range phasing, whereas for the Omni series of chips, 642,079 SNPs were included. The final set of SNPs used for long-range phasing was composed of 785,863 SNPs.

Whole-Genome Sequencing and Imputation
Sequence data were obtained from about 2,230 Icelanders using methods described previously by Holm et al. (2011). Sequencing was carried out to an average depth of at least 10×. This resulted in the identification of approximately 38.5 million SNPs and small indels that were available for imputation. Long-range phasing, imputation, and association testing (by logistic regression) were done as described previously (Kong et al., 2008, 2009; Holm et al., 2011; Stacey et al., 2011). For conditional analysis of the IRF4 region, the rs12203592-T allele count of each individual was given as a covariate in the logistic regression.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.10.022.

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