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A heterozygous c-Maf transactivation domain mutation causes congenital cataract and enhances target gene activation

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MAF, one of a family of large Maf bZIP transcription factors, is mutated in human developmental ocular disorders that include congenital cataract, microcornea, coloboma and anterior segment dysgenesis. Expressed early in the developing lens vesicle, it is central to regulation of lens crystallin gene expression. We report a semi-dominant mouse c-Maf mutation recovered after ENU mutatgenesis which results in the substitution, D90V, at a highly conserved residue within the N-terminal 35 amino-acid minimal transactivation domain (MTD). Unlike null and loss-of-function c-Maf mutations, which cause severe runting and renal abnormalities, the phenotype caused by the D90V mutation is isolated cataract. In reporter assays, D90V results in increased promoter activation, a situation similar to MTD mutations of NRL that also cause human disease. In contrast to wild-type protein, the c-Maf D90V mutant protein is not inhibited by protein kinase A-dependent pathways. The MTD of large Maf proteins has been shown to interact with the transcriptional co-activator p300 and we demonstrate that c-Maf D90V enhances p300 recruitment in a cell-type dependent manner. We observed the same for the pathogenic human *NRL* MTD mutation S50T, which suggests a common mechanism of action.

INTRODUCTION

Congenital cataract affects between two and five per 10 000 live births (1) with inherited autosomal dominant forms accounting for around half (2). Age-related cataract, the commonest preventable form of blindness, has a strong but undetermined genetic component (3–6). The genes underlying congenital cataract—particularly those associated with progressive phenotypes—may be viewed as potential candidates for age-related forms (7,8). Genes underlying congenital cataract have been identified successfully using positional and candidate approaches describing defects in lens-expressed cytoskeletal and membrane proteins, crystallins and transcription factors (9). However, the genetic basis remains undefined in the majority of cases. In parallel, murine mutagenesis programmes have identified a wide range of dominant cataract mutants in many of the orthologous genes underlying human phenotypes, providing excellent models for human cataractogenesis (10–12).

We have previously identified *MAF* as a gene associated with human disease, including progressive forms of congenital cataract, microcornea, coloboma and anterior segment dysgenesis (13,14). Homozygous null mutant *c-Maf* mouse embryos have defective lens formation, small eyes and decreased expression of crystallins (15–17). While heterozygous null mice exhibit normal eye development, a single semi-dominant mutation (designated *Ofl*, opaque flecks in lens: R291Q) within the DNA-binding domain is associated with congenital cataract in heterozygotes (11). C-Maf is expressed early in the developing lens vesicle and is central to the regulation of lens crystallin gene expression in the differentiating primary posterior lens fibres (15,18). The protein acts both as a homo- and heterodimer and binds to maf response elements (MAREs) (19,20).

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C-Maf is one of a subfamily of bZIP transcription factors which carry a basic DNA-binding motif and a leucine zipper to allow dimerization (21). The Maf proteins exist as both small and large isoforms. The small Maf proteins (MafF, MafG and MafK) lack a transactivation domain and dimerise with CNC (cap 'n' collar) and/or Bach proteins, whereas the large Maf proteins (c-Maf, MafB or KRML, MafA or L-Maf, and NRL) have an N-terminal transactivation domain (20,22–24). In addition to *MAF*, only mutations of *NRL* are associated with human disease. Missense *NRL* mutations within the transactivation domain have been shown to cause dominant retinitis pigmentosa (25). Most recently, null mutations in NRL have been described in autosomal recessive retinitis pigmentosa (26). Although the functions of the c-Maf DNA-binding domain have been closely studied, those of the transactivation domain remain less well characterized. As with other large Maf proteins, both Nrl and c-Maf carry a PST-rich (proline serine threonine) domain that is N-terminally located. It has been reported that, for Nrl, a 35-amino-acid minimal transactivation domain (MTD) within this PST-rich domain is sufficient for transactivation of target promoters (27). This MTD has been shown to interact with TATA-binding protein (TBP) *in vitro*, whereas *in vivo* TBP has been demonstrated to be part of multi-protein complexes that contain c-Maf and Nrl (27).

We report a murine c-Maf mutation (termed ENU424) recovered in a random mutagenesis screen following paternal exposure to ethylnitrosourea. The dominant cataract phenotype results from a D90V mutation of a highly conserved residue within the 35-amino-acid MTD. The phenotype of the D90V homozygote is isolated cataract. This is distinct from those caused by deletion or by loss-of-function mutations which cause extraocular phenotypes such as severe runting, renal abnormalities and neonatal death (11,16,17). In reporter assays, the D90V missense mutation results in increased activation of the Pitx3 promoter, a result which parallels observations for the transactivation domain mutations of NRL. The c-Maf transactivation domain is reported to interact with the N-terminal region of the transcriptional co-activator p300, and we therefore measured this interaction using a mammalian two-hybrid approach. We demonstrate that the transactivation domain of NRL has identical p300 interaction properties as c-Maf, and that both the c-Maf D90V and the human *NRL* mutation S50T enhance recruitment of p300, suggesting a common mechanism of action. Finally, we demonstrate that transactivation by the c-Maf D90V mutation, unlike the wild-type protein, is not inhibited by protein kinase A (PKA) dependent pathways.

RESULTS

Preparation and genetic mapping of murine cataract mutant ENU424

The original mutant animal was found among the offspring in a mutagenesis experiment at the Institute of Mammalian Genetics, Neuherberg. Wild-type (C3H/El × 102/El) F1 males were exposed to 250 mg ENU/kg body weight before they were mated at such an interval afterwards that treated spermatogonial stem cells were sampled (28). The mutant animal, assigned the lab symbol ENU424, expressed a granular nuclear opacity. The genetic nature of the variant phenotype was confirmed in a transmission-breeding test in which the animal was mated to homozygous wild type and offspring with a similar pulverulent cataract phenotype was produced (29). The mutation was tested further and shown to be fully penetrant with no viability defects as a heterozygote. Homozygotes were shown to be viable and fertile (30). The semi-dominant phenotype associated with the ENU424 mutation is documented in Figure 1. Mutant heterozygotes express a mild granular nuclear opacity and homozygotes express a more dense and severe nuclear opacity. There is no effect of the mutation on lens or eye size.

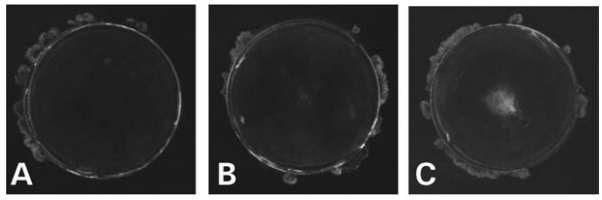

Figure 1. Lens phenotype of ENU424 mice. Lens phenotype in 5-week-old littermates in +/+ (**A**), ENU424/+ (**B**) and ENU424/ENU424 (**C**) mice.

The mutation ENU424 was mapped to Chr 8 with the following gene order and genetic distances between markers (the number of crossovers/number of animals genotyped for adjacent loci given in parentheses): D8Mit129-(10/97)-D8Mit305-(18/97)-D8Mit113-(5/95)-D8Mit271-(4/95)-D8Mit 167-(6/97)-ENU424-(10/97)-D8Mit280. These results localize the ENU424 mutation to the Chr 8 region containing the *c-Maf* gene.

Nature of the Maf mutation in ENU424

C-Maf mRNA encodes a 370-amino-acid polypeptide. The coding region was screened for mutations using direct sequencing on genomic DNA from homozygous, heterozygous and control animals on the C3H/El and 102/E1 backgrounds. Sequencing revealed an A to T transversion at nucleotide position 269 of *c-Maf* (GenBank accession no. S74567). This mutation substitutes an aspartic acid to valine at amino-acid position 90 (D90V) in the N-terminal transactivation domain of c-Maf (Fig. 2). This residue is also highly conserved in the human and murine Mafb. This sequence variant was neither present in the C3H/El nor 102/El control lines. There was no further *c-Maf* coding sequence abnormality. Hence, we designate the mutant allele symbol Maf^{ENU424}.

Transactivation of Pitx3 promoter by Maf

It has previously been demonstrated in the rat that c-Maf regulates its own promoter (31) as well as that of a range of crystallin genes including the αa crystallin gene, Cryaa, in the mouse (32). Since previous reports (33) have demonstrated that 1 kb of the proximal promoter of *Pitx3* may contain as many as four potential Maf recognition elements (MAREs), we postulated that expression of lens-derived Pitx3 might require transactivation by the Maf transcription factor. To test this prediction, we designed experiments to compare the

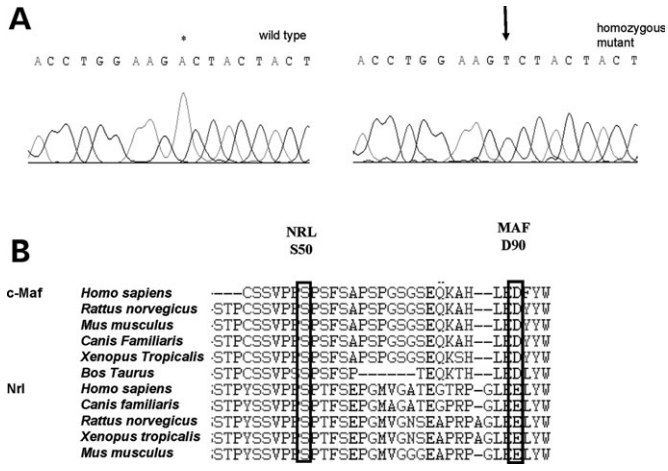

Figure 2. C-Maf mutation (**A**) Sequence comparison between wild type (left) and mutant (right) murine c-Maf sequence. A to T transversion at nucleotide position 269 is arrowed. (**B**) Amino acid sequence conservation of c-Maf and Nrl MTDs. Alignments demonstrate high conservation of the invariant aspartic acid residue at position 90 in c-Maf. Although the residue is not precisely conserved in Nrl, the substitution of aspartic acid for glutamic acid shows functional conservation. However, the serine 50 and proline 51 residues which, in NRL, are known to be mutated in autosomal dominant retinitis pigmentosa are highly conserved between the two large Maf proteins.

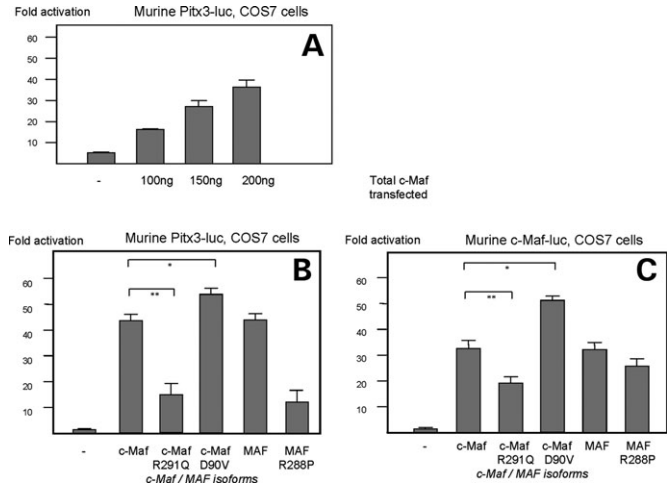

Figure 3. Transactivation of *c-Maf* and *Pitx3* promoters by c-Maf. (**A**) COS-7 cells were transfected with *Pitx3* pGL3 luciferase reporter and different amounts of plasmid encoding wild-type c-Maf, which activated it in a dose-dependent fashion. Activation of *Pitx3* promoter (**B**) and *c-Maf* promoter (**C**) by wild-type c-Maf compared to murine c-Maf mutants D90V and R291Q (11). Human wild-type MAF was compared to human R288P mutation (13). For both the *c-Maf* and *Pitx3* promoters the D90V mutation demonstrated a significantly stronger activating ability than wild type while both the c-Maf R291Q and MAF R288P demonstrated significantly reduced activation. Fold activation is expressed relative to the Luc/β-gal ratio seen after co-transfection of reporter with the empty expression vector which has been arbitrarily set as 1. $^{*}P < 0.0001$; $^{**}P < 0.001$.

abilities of c-Maf to transactivate the *Pitx3*, *Maf* and *Cryaa* promoters. We subcloned each of these promoter DNA fragments into the pGL3 basic luciferase vector for transient transfection studies. The precise transcription start site of c-Maf is not known in mouse. However, since the murine and rat promoters show 92% homology, we cloned the upstream mouse sequence that is homologous to the region in rat that has been shown to contain MARE sites and which has been shown to be implicated in binding MAF for autoregulation. Importantly, the MARE and CRE sites identified in this region in the rat are 100% conserved in the mouse implying functional conservation. C-Maf is known to have an important role in renal development and maintenance. Therefore, COS7 cells (African Green Monkey kidney fibroblast), which we showed to express MAF, were chosen to co-transfect with each reporter and with plasmids encoding different alleles of the c-Maf transcription factor. As previously demonstrated c-Maf transactivated the *Cryaa* and *c-Maf* promoters in a dose-dependent fashion (data not shown) and the murine *Pitx3* promoter (Fig. 3A). It has been shown that lens and pituitary precursors have comparable gene expression and in particular Pitx3 is required for both lens vesicle and pituitary pre-placode formation and cell specification (34). Therefore, this result was also confirmed in the murine pituitary corticotroph cell line ATt20 (Fig. 4A), which, like COS7 cells, express c-Maf.

The dominant transactivation domain D90V mutation increases Maf activity

We then determined the ability of different mutant isoforms of the c-Maf transcription factor to transactivate the *Pitx3, c-Maf* (Fig. 3B and C) and confirmed this for the *Cryaa* promoter (data not shown). In addition to the wild-type murine c-Maf control, we assayed the ENU424 (D90V) allele and the *opaque flecks in lens* allele (DNA-binding domain mutation R291Q) that we have previously reported. We have previously demonstrated that the murine R291Q mutation within the DNA-binding domain significantly reduces DNA binding in a sequence-dependent manner (13). Unsurprisingly, therefore, this R291Q mutant has a substantially reduced transactivation ability at all promoters tested ($P < 0.0001$ in all cases) when compared to the murine wild-type c-Maf, although the effect is greater for the *Pitx3* promoter than for that of *c-Maf*. On all murine promoters, we found that the transactivation potential of wild-type human MAF was similar to the murine orthologue. Furthermore, like the R291Q mouse mutation, the human congenital cataract-causing DNA-binding mutation R288P also exhibited substantially reduced transactivation capacity (13). By contrast with loss-of-function mutations, the D90V c-Maf isoform has significantly increased transactivation ability at all three promoters ($P < 0.0001$). Interestingly, at the *Pitx3* promoter, the enhanced activation ability of the D90V c-Maf isoform relative to wild type was significantly greater in ATt20 cells than in COS7 cells (Figs 3 and 4), whereas the difference was less marked on the c-Maf promoter, suggesting that in regulating some of its target genes c-Maf is likely to act in conjunction with other proteins expressed in a cell lineage-specific manner as part of a complex.

Large Maf transactivation mutation enhances interaction with p300

It has previously been shown (32) that the transactivation domain of c-Maf interacts directly with p300 and that this

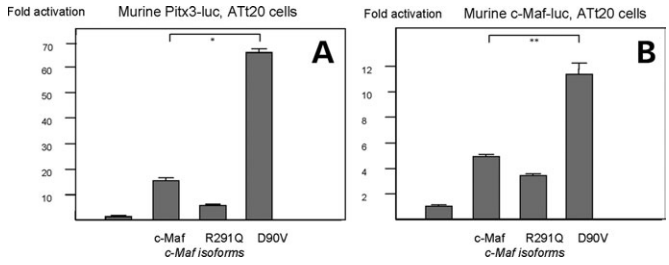

Figure 4. Transactivation of *Pitx3* (**A**) and *c-Maf* (**B**) promoters by murine isoforms of Maf in ATt20 cells. There is an increased activity of the D90V mutation when compared to wild type and, when compared to COS7 cells this differential activity is substantially increased. This suggests a cell type-specific effect (Lane 1: Empty expression vector. Lane 2 Wild-type murine c-Maf. Lane 3 R291Q isoform, murine c-Maf. Lane 4 D90V variant, murine c-Maf). $^{*}P < 0.0001$; $^{**}P = 0.0005$.

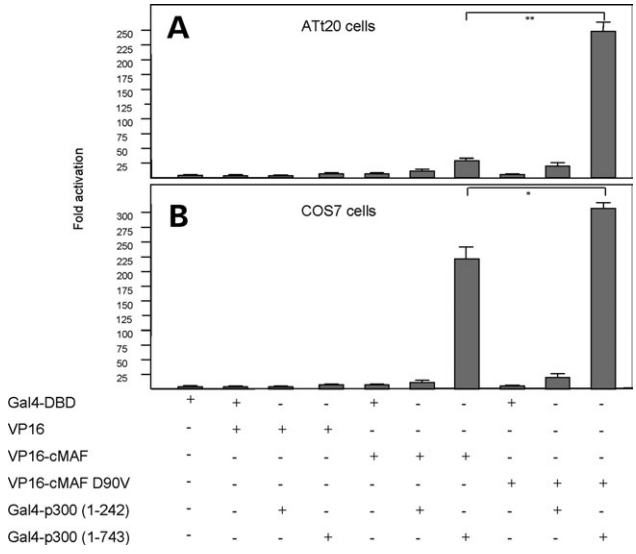

Figure 5. Mammalian two-hybrid analysis confirming interaction of c-Maf with p300. (**A**) Murine ATt20 cells (**B**) COS-7 cells. Cells were co-transfected with a pG5E1b-luciferase reporter with plasmids encoding the Gal4 DNA-binding domain (either alone or fused to various regions of p300) and with a plasmid expressing the VP16 acidic activation domain (again either alone or fused to c-Maf). Expression of the luciferase reporter is dependent upon interaction between Gal4-p300 and VP16-c-Maf chimeras to recruit VP16 to the E1b-TATA promoter to initiate transcription. Co-expression of the amino terminus of p300 (residues 1–743; lane 7) together with VP16-Maf significantly increased luciferase activity in both ATt20 and COS7 cells. In both cell lines, the activity of the D90V mutant N-terminus (lane 10) has a significantly increased activity compared to wild type. Fold activation is expressed relative to luciferase/growth hormone activity obtained after co-transfection of the specific reporter (G5E1b reporter) and an empty expression vector. $^{*}P = 0.015$; $^{**}P < 0.0001$.

interaction is via the p300 amino-terminus, specifically a region between residues 180 and 662 containing the zinc-finger and CREB-binding domains. We examined the effect of the c-Maf D90V activating mutation on this interaction, using a mammalian two-hybrid approach (Fig. 5). Two p300 amino-acid sequences (1–242 and 1–743) were fused to the Gal4 DNA-binding domain, while the c-Maf N-terminal 139 amino acids, containing the transactivation domain were fused to the VP16 MTD. We confirmed that the expression of the UAS (upstream activating sequence) reporter required the presence of both the fusion constructs, thereby confirming that there is indeed recruitment of p300 to MAF. When the identified mutant alleles of c-Maf were examined in the same assay, we observed a marked enhancement of recruitment of p300 with the gain-of-function D90V mutant allele. The mutant D90V c-Maf transactivation domain had a greater impact on p300 recruitment in the ATt20 cell background, than in the COS7 cells, again suggesting cell type specific effects (Fig. 5).

Of other large MAF proteins, NRL is known to be mutated in autosomal dominant and recessive retinal dystrophies. Among the mutations known to cause dominant disease, the majority affects two residues, S50 (S50T, S50P and S50L) and P51 (P51T) (35) that are highly conserved between MAF and NRL (Fig. 2). The S50T isoform of NRL has previously been shown to have a significantly enhanced ability to transactivate the rhodopsin promoter when compared with the wild-type protein (25). We therefore tested whether the dominant, activating NRL mutation S50T in the transactivation domains of NRL might also enhance the ability of NRL to interact with p300. We attached the NRL transactivation domain contained within the N-terminal 99 residues (both mutant P50T and wild type) to VP16 and confirmed not only that the transactivation domain of NRL interacts with the same region of p300 in a mammalian two-hybrid system but also that, like the D90V c-Maf mutation, the S50T NRL mutation enhances this interaction (Fig. 6).

Maf transactivation and signal transduction

It has previously been shown that, in activating the crystallin γD promoter, c-Maf transactivation capacity was regulated by PKA and PKC (36). Therefore, the effect of regulating protein kinase activity on the activity of wild-type and mutant c-Maf was explored. We confirmed that forskolin strongly inhibited transactivation of the *c-Maf* (Fig. 7) and *Pitx3* (data not shown) promoters by c-Maf and that the MAPK/ERK antagonist PD98059 increased transactivation (Fig. 7A). Importantly, when these effects were examined on the murine mutant D90V the forskolin inhibition was largely abolished (Fig. 7B and C).

DISCUSSION

This work provides both genetic and functional evidence that the *ENU424* mutant, which is associated with isolated congenital cataract, involves mutation of the *c-Maf* transcription factor. We have demonstrated, by genetic mapping, that the *ENU424* locus lies close to the *c-Maf* gene and have identified a mutation (D90V) of a highly conserved residue within the c-Maf MTD. This mutation is co-dominantly expressed and we have shown that the amino-acid substitution enhances transactivation via an alteration in the ability to interact with p300, demonstrating a potential mechanism for the apparent gain of function.

Through translocation breakpoint mapping and subsequent mutation analysis, *MAF* gene involvement has been identified in human cataract families with pulverulent cataract and cerulean cataract (13,14). In two families, one with a hypomorphic

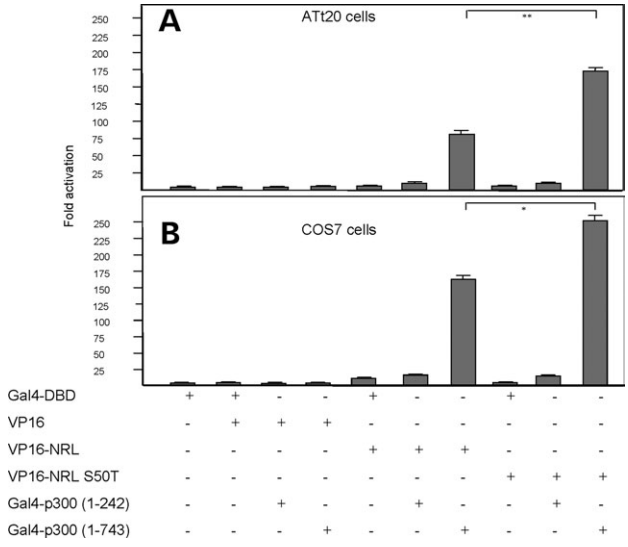

Figure 6. Mammalian two-hybrid approach demonstrating interaction of N-terminus of NRL with p300. (**A**) Murine ATt20 cells, (**B**) COS-7 cells. Cells were co-transfected with a pG5E1b-luciferase reporter with plasmids encoding the Gal4 DNA-binding domain (either alone or fused to various regions of p300) and with a plasmid expressing the VP16 acidic activation domain, again either alone or fused to NRL, as indicated. Expression of the luciferase reporter is dependent upon interaction between Gal4-p300 and VP16-NRL chimeras to recruit VP16 to the E1b-TATA promoter to initiate transcription. Co-expression of the amino terminus of p300 (residues 1–743; lane 7) together with VP16-NRL significantly increased luciferase activity in both cell types. In both cell lines, the activity of the S50T mutant N-terminus (lane 10) has a significantly increased activity compared to wild type. Fold activation is expressed relative to luciferase/growth hormone activity obtained after co-transfection of the specific reporter (G5E1b reporter) and an empty expression vector. $^{*}P = 0.0009$; $^{**}P < 0.0001$.

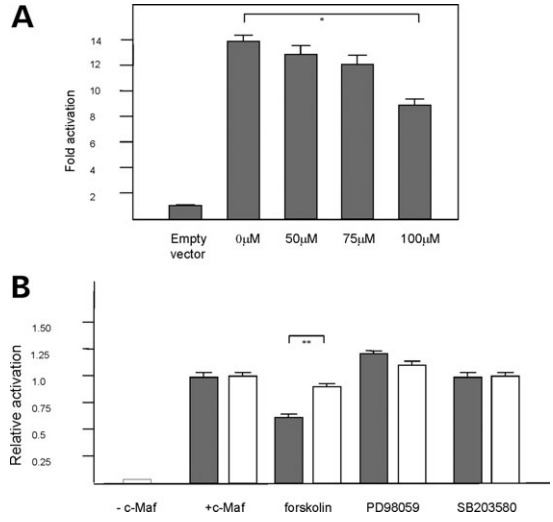

Figure 7. Effect of signal transduction regulators on c-Maf transactivation of c-Maf promoter. All experiments performed on ATt20 cells. (**A**) Dose response curve for forskolin (PKA activator). Transactivation reduced by 100 μM forskolin. (**B**) Cells were transfected with the *c-Maf* promoter luciferase construct and an expression construct for wild-type c-Maf (left) and D90V c-Maf mutants (right) as described in Materials and Methods. Compounds added as indicated at the following concentrations: forskolin, 100 μM; PD98059, 20 μM; SB203580, 10 μM. Only the forskolin showed a significant difference between wild type and D90V mutant (as indicated by **). (Transactivation normalized to transfected cells treated with vehicle only). $^{*}P = 0.0015$; $^{**}P = 0.0007$.

R288P DNA-binding mutation and the second with a K297R DNA-binding domain mutation, additional phenotypic features included microcornea and coloboma. This underlines the role of lens-derived signalling for the development of the anterior segment of the eye and in closure of the anterior portion of the optic fissure. In all families, the human cataract phenotypes, although of early onset, are progressive in nature and affected individuals tended to have surgery in mid to late childhood or even in adult life. Thus, such defects not only alter lens development but also disrupt the maintenance of lens clarity. The study of MAF may therefore give insights into both congenital and later-onset forms of cataract both of which are known to have a strong genetic component (7,8).

Mutagenesis experiments in mice have led to the production of a large resource, with approximately 200 murine dominant cataract mutants generated (12). Many have been mapped and mutations in genes for crystallins, the lens protein *Lim2*, the lens-specific gap junction protein *Gja8* and the transcription factors *Pax2*, and *Pax6* all provide excellent mammalian models for human cataractogenesis (12). This is the second ENU-derived mutation of c-Maf that is to be described in the mouse. We have previously shown that a hypomorphic mutation, R291Q (Maf^{ofl}), within the DNA-binding domain of c-Maf has a phenotype in homozygous mutants which strongly resembles that of c-Maf null homozygotes (11). Maf^{Ofl}/Maf^{Ofl} homozygotes and those homozygous for null alleles have additional non-ocular manifestations which are absent in mice carrying the D90V c-Maf isofom. These include reduced survival, runting and also tubular nephritis. The mechanism by which *c-Maf* mutation produces extraocular manifestations is not known although as *c-Maf* is widely expressed that it is likely to have extraocular functions (15) and has been implicated in renal development (37). Therefore, the different phenotypic manifestations of the ENU424 mutation suggest that this mutation does not act as a simple hypomorphic allele.

We have used *in vitro* luciferase reporter assays to confirm that c-Maf transactivates the *c-Maf* promoter and to demonstrate that it is active at the *Pitx3* promoter. Like MAF, PITX3 is mutated in human forms of congenital cataract and demonstrates significant phenotypic heterogeneity (13,14, 38,39) in particular causing severe forms of anterior segment dysgenesis, including Peters anomaly. In the mouse absence of *Pitx3* expression results in the *aphakia* phenotype in which there is grossly disrupted anterior ocular development due to defective lens development (33). The phenotype is not strictly primary aphakia since at around 10.5 days of embryonic life the early lens vesicle fills with cells from the lens epithelium but subsequently fails to detach correctly from the surface epithelium. The lens vesicle does not produce primary and secondary lens fibre cells and its development is arrested. Subsequent resorption results in dissolution of the vestigial lens and anterior segment disorganization. In mice lacking c-Maf, failure of elongation of the posterior lens fibres as a result of reduced crystallin expression results in the arrest of lens vesicle development and the resulting hollow lens vesicle collapses, causing a secondary aphakia with reduction in globe size (microphthalmia). This overlap in both murine and human

phenotypes combined with the observation that both c-Maf and Pitx3 are co-expressed in the developing vesicle, even though Pitx3 is expressed earlier from E10 in the lens placode, is consistent with an interacting role for both in the organization of early lens development.

The promoter activities of both human (R288P) and murine (R281Q) mutations are significantly reduced when compared with wild type, as expected from disrupted DNA-binding domains. There is a five-fold increase in the activity of the ENU424, D90V, mutation at the *Pitx3*, and also at the *c-Maf*, promoters. This suggests that the D90V mutation is an activating mutation and is consistent with the semi-dominant effect on phenotype. The D90V substitution occurs at a conserved aspartic acid residue, within the highly conserved N-terminal transactivation domain that characterizes the large Maf proteins such as c-Maf, Nrl and MafB. The aspartic acid residue is conserved in c-Maf proteins among the vertebrates from human to Xenopus while the homologous residue in NRL, glutamic acid, is functionally similar. The ENU424 D90V substitution would replace an acidic residue with a neutral and hydrophobic residue. This will remove a surface polar residue with hydrogen bonding capability, and replace it with a residue likely to be buried within the protein core. However, there are currently no crystal structures to guide further hypotheses of D90 function.

The enhanced activation ability of the D90V c-Maf isoform over the wild type at both the *c-Maf* and *Pitx3* promoters was dependent upon cell type suggesting that it is likely to act as part of a broader multi-protein complex. In the transcriptional regulation of αB and γF crystallin gene expression, it has now been demonstrated that the binding sites of c-Maf and Pax6 may overlap (40,41) and, using chromatin immunoprecipitations, that Pax6 and c-Maf interact *in vivo* in lens cells. Furthermore, c-Maf has been shown to act synergistically with Sox family proteins, including Sox2 and Sox3 and that it is the amino terminus of c-Maf that mediated this interaction (42).

The c-Maf N-terminal transactivation domain, which includes D90, has been shown to interact with an N-terminal region of p300 between amino acids 180 and 662 that includes the zinc-finger and the CREB-binding domain. Furthermore, with Pax6 and c-Maf, CREB has been previously implicated in the regulation of αA crystallin regulation though regulation of chromatin remodelling (43). We confirmed the c-Maf/p300 interaction using a mammalian two-hybrid approach through co-expression of GAL4-p300 (residues 1–743) with VP16-cMaf (either wild-type or D90V mutant forms) and demonstrated that D90V enhanced this interaction. The majority of the dominant NRL mutations that cause retinitis pigmentosa reside in its homologous transactivation domain and affect two residues, serine at position 50 and proline at position 51. Their precise functional consequences are unknown although S50T has a significantly increased ability to transactivate the rhodopsin promoter, both in isolation and in combination with the retinal-specific transcription factor CRX. Given the homology between the orthologous domains of NRL and MAF, we tested interaction of the NRL N-terminal domain with the identical domains of p300. This confirmed the potential interaction and also suggested that, like the D90V c-Maf mutation, the S50T mutation of NRL enhances promoter activation. This points to a potentially common mechanism of action of mutations in the two molecules. The 35-amino-acid MTD has previously been shown to interact with TBP, as has Pax6 (27,44). It has been suggested that c-Maf and Pax6 may recruit TBP to control regions of the αA crystallin promoter and it remains possible that mutations in the MTD of large Maf proteins act to enhance this recruitment.

Previously, it has been demonstrated that c-Maf is regulated by PKA and PKC signal transduction pathways. Civil *et al*. (2002) have demonstrated that in CHO cells the ability of c-Maf to activate the crystallin γD promoter was negatively regulated by forskolin (a PKA activator) and 12-O-tetradecanoylphorbol 13-acetate (TPA) (36). We also find that activation of PKA inhibits transactivation by c-Maf, and shows that the D90V ENU424 mutation abolishes the ability of both forskolin and PD98059, an inhibitor of MAPK/ERK, to modulate c-Maf activity. The inhibitory phosphorylation site was not identified by Civil *et al*. (2002), although its presence either within the putative PKA/PKC site at residues 288–293 or within the putative tyrosine kinase phosphorylation region (RLVRERDAY between amino acids 333–341) was excluded (36). Phosphorylation (by p38 MAPkinase) of residues Thr57 and Thr113 of the transcriptional activating domain of MafA has been confirmed and has also been suggested for the corresponding residues (residues 62 and 109) in the conserved regions of c-Maf. However, SB203580, a specific inhibitor of p38 MAP kinase had no effect on the relative activation of c-Maf (45). It is quite likely that the site phosphorylated lies either in p300, or another recruited co-modulator, as has been described for other transcription factors (46).

The identification of allelic mutational series is a powerful tool to study molecular function. We have described the first c-Maf transactivation domain mutation, a gain-of-function substitution expressed in a co-dominant manner. We have shown *in vitro* that substitution of D90 by valine appears to alter both the transactivation ability of c-Maf and its ability to recruit p300 in a cell-specific fashion. Such observation will now need to be supplemented by *in vivo* analyses, in both heterozygous and homozygous mice, of genes regulated by c-Maf. Unlike other murine c-Maf mutations, the D90V substitution does not result in extraocular manifestations, specifically lacking the renal manifestations we observed with a dominant R288P DNA-binding domain mutation. This suggests that investigation of tissue-specific (e.g. renal) interactors of c-Maf will be important in elucidating its function.

MATERIALS AND METHODS

Mouse breeding

Prior to the initiation of the present mapping and breeding studies, a congenic C3H/El-ENU424 mutant line was established. For mapping, a homozygous mutant C3H/El-ENU424 mouse was outcrossed to wild-type strain C57BL/6El, heterozygous C3B6-ENU424/+ offspring were recovered and backcrossed to wild-type C57BL/6El mice. The backcross offspring were phenotyped for the ENU424 mutation by slit lamp

biomicroscopy and genotyped for our standard microsatellite marker panel as previously described (47). Upon localization to Chr 8 mice were genotyped for additional markers in the critical region. Segregation data were analysed with Map Manager Version 2.6.5 (48) and the gene order was determined by minimizing the number of multiple crossovers. Animals were bred and maintained according to the German law for the protection of animals. All inbred strain C3H/El and C57BL/6El animals were obtained from breeding colonies maintained by the GSF-Department of Animal Resources at Neuherberg.

Scoring for cataracts

Mice were ophthalmologically examined at 3 weeks of age by slit lamp biomicroscopy as previously described (29).

Mutation analysis

Mice were euthanized at 5 weeks of age by cervical dislocation, dissected and liver samples were snap frozen on dry ice. The liver samples were homogenized in the presence of proteinase K and incubated at 37° overnight. Genomic DNA was extracted by a standard phenol/chloroform separation in an AutoGen540 automatic DNA extractor. PCR amplification of the *c-Maf* gene was performed as follows (primers on request); DNA (40 ng) was suspended in a 20 μl reaction containing 10 pmols of each forward and reverse primer, 0.75 mM dATP, dGTP, dCTP, dTTP, 67 mM Tris–HCl (pH 8.0), 3.7 mM $MgCl_2$, 6.7 μM EDTA, 16 mM $(NH_4)_2SO_4$ 0.085 mg/ml BSA and 0.1 units of *Taq* DNA polymerase. Due to the GC sequence content of this gene, some reactions required the addition of enhancing agents either DMSO at 10% or both DMSO at 10% and Betaine at 1 M final concentration. Samples were processed through 30 cycles of amplification consisting of 45 s at 94°C (denaturation), 45 s at 58°C (annealing) and 1 min at 72°C (extension). In the last cycle, the final step was lengthened to 10 min. Direct sequencing of PCR products was performed using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on a fluorescent sequencer (ABI 377) in accordance to the manufacturer's instructions.

Plasmids for transfection

Reporter plasmids. Promoters were amplified from mouse genomic DNA using the following primers: *Pitx3* promoter gcggcgttctggagcgcaggcag and gtaacccgcctgtcatactccttg (1140 bp) and *c-Maf* promter gctccgctgcgcgctttgcataag and gatgcagaggatggatcaaagatcg (1026 bp). Promoter-luciferase constructs were made by excising promoter fragments from the PCR cloning vector pCR-Blunt II TOPO (invitrogen) and inserting into the polylinker of pGL3.

Expression constructs. Constructs were generated through using the universal cloning based from Gateway®Technology. Essentially *att*B PCR primers were designed to amplify the cDNA sequence in accordance to Gateway Technology guidelines. Primers also included a FLAG tag sequence. *Att*B PCR products were generated for full-length, wild-type mouse *c-Maf* and human *MAF* genomic gene, as well as murine mutants Ofl (R291Q) and ENU424 (D90V) and the human mutant isoform R288P. Primer sequences: mouse c-Maf forward: 5′ GGGGACAAGTTTGTACAAAAAAGCAGGCT CCACCATGGATTACAAGGATGACGACGACGACAAGG CTTCAGAACTGGCAATGAAC; mouse c-Maf reverse: 5′ GGGGACCACTTTGTACAAGAAAGCTGGGTCACATGAA AAATTCGGGAGAGGA; human MAF forward 5′ GGGG ACAAGTTTGTACAAAAAAGCA GGCTCCACCATGGATT ACAAGGATGACGACGACGACAAGGCATCAGAACTGG CAATGAGC; human MAF reverse: 5′ GGGGACCACT TTGTACAAGAAAGCTGGGTCACATGAAAAACTCGGGA GAGGA.

BP reactions were then performed to transfer to the *att*P-containing vectors pDONR 221 (Invitrogen). LR reactions were then carried out, according to manufacturer's instructions, to transfer the genes of interest into an attR-containing destination vector to create the *att*B-containing expression clone. Inserted gene sequences were then transferred across, again according to manufacturer's instructions, into a Gateway®-compatible destination vector pcDNA-DEST40 (Invitrogen) to generate expression constructs.

Mammalian two-hybrid constructs. Transactivation domain elements were amplified from initial pDONR221 constructs for the wild-type mouse c-Maf and ENU424 mutant construct and the equivalent transactivation domain for NRL using Gateway® 5′ modified forward and reverse c-maf transactivation primers (first 139 amino acids) forward: 5′ GGGGACAA GTTTGTACAAAAAAGCAGGCTCCACCATGGATTACAA GGATGACGACGACGACAAGGCTTCAGAACTGGCAAT GAACAATTCCG; reverse: 5′ GGGGACCACTT-TGTACA AGAAAGCTGGGCCTAGGCCAGCTGCTGCGCTCCCCGCG and Gateway 5′modified forward and reverse NRL transactivation domain primers (first 99 amino acids) Forward: 5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAACAT GGATTACAAGGATGACGACGACGACAAGGCCCTGCC CCCCAGCCCCCTGGC Reverse:5′ GGGGACCACTTTGTA CAAGAAAGCTGGGCTCACAGCAGCTCCATGGCCTCTT CAGGAC.

For NRL, mutagenesis was performed using the QuickChange kit (Stratagene) with the following primer: NRL (P51L), 5′ CTTACAGCTCAGTGCCTCCTTCACTCACCTT CAGTGAACCAGGC (only sense sequence given), under manufacturer's guidelines. For the mammalian two-hybrid experiments, the vector pVP16 was Gateway® converted to a Gateway® destination vector by ligating a blunt-ended cassette containing *att*R sites flanking the *ccd*B gene and the chloramphenicol resistance gene into the multiple cloning site of the vector. LR reactions were then performed between the created entry clones and the GatewayR-converted vector containing the *att*R sites to create the expression constructs, according to manufacturer's instructions. The following GAL4 fusion domain plasmids were kindly provided by Dr S. Bhattacharya, GAL4-p300 (1–242) and GAL4-p300 (1–743). The effect the various constructs gave on binding was measured using expression of luciferase driven by five GAL4-binding sites in pGLuc5 (provided by Prof. A. Sharrocks).

Cell culture and transfection

COS7 and ATt20 (kindly provided by A. Warhurst) cells were cultured in DMEM with Glutamax (Invitrogen life sciences), and 10% fetal calf serum. Cells were harvested and seeded into 24 well plates to a cell density of 1×10^4 cells per ml. Transfections were performed using lipofectamine plus reagent as outlined in the supplier's instructions (Invitrogen). Typically, 750 ng of reporter construct was transfected with 250 ng of expression construct and 0.5 μg of CMV-gal. After 6 h, the transfection medium was replaced by complete medium containing 10% fetal calf serum. After 24 h, the media was removed, cells were lysed and luciferase reporter assays were performed on lysates using the luciferase assay system (Promega) according to the manufacturer's instructions using a luminescent plate reader. Reporter gene activity was normalized for differences in transfection efficiency on the basis of activity of co-transfected CMV-βgal by performing a standard β-gal assay, as previously described (49). Mammalian two-hybrid experiments were performed as previously described (50) in accordance to manufacturer's guidelines (Promega). Luciferase expression was normalized to β-gal, as previously described (49). Transfections were performed in triplicate and replicated with similar results in at least three independent experiments. Results are the mean ± SD from three individual experiments. All statistical analyses were performed with two-tailed Student's *t*-tests and considered significantly different for $P < 0.05$. Again all experiments were performed in triplicates and on at least three separate occasions.

For signal transduction agonists and antagonists, ATt20 cells were co-transfected with pGL3–Pitx3 promoter construct and expression construct for c-Maf. Signal transduction ant(agonists) were added when supplementing the cells with complete medium 6 h after transfection at varying concentrations as indicated at the following concentration ranges forskolin, 0–100 μM; PD98059 0–20 μM; SB203580, 0–10 μM.

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Conflict of Interest statement. None declared.

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