Loss of MAFB function in human and mouse causes Duane syndrome, aberrant extraocular muscle innervation, and inner ear defects

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SUMMARY

Duane retraction syndrome (DRS) is a congenital eye movement disorder defined by limited horizontal gaze and globe retraction. While studies of adults with DRS have reported abducens nerve hypoplasia and aberrant innervation of the lateral rectus muscle by axons of the oculomotor nerve, this pathology has not been confirmed in an animal model and the developmental etiology of DRS remains unclear. Here, we report three loss-of-function *MAFB* mutations causing DRS and a dominant-negative *MAFB* mutation causing DRS and inner ear defects, and propose a threshold model for variable loss of MAFB function. We confirm human DRS pathology in *Mafb* knockout mice, and demonstrate that disrupting abducens nerve development is sufficient to cause secondary aberrant innervation of the lateral rectus muscle by the oculomotor nerve. This suggests that a wide variety of clinical insults to the developing abducens nerve will cause DRS, and provides an animal model to study aberrant innervation.

INTRODUCTION

Duane retraction syndrome (DRS) is the most common of the congenital cranial dysinnervation disorders with a prevalence of 1:1000 (Engle, 2010). Affected individuals have unilaterally or bilaterally limited eye abduction and globe retraction with attempted eye adduction. Postmortem brainstem and orbital examination of two adults (Hotchkiss et al., 1980; Miller et al., 1982) and magnetic resonance imaging of four patients (Demer et al., 2007) with DRS revealed absence or hypoplasia of the abducens nerve, which normally innervates the lateral rectus (LR) extraocular muscle to abduct the eye, and were consistent with aberrant LR muscle innervation by axons of the oculomotor nerve, which normally innervates the medial rectus (MR), inferior rectus (IR), superior rectus (SR), and inferior oblique (IO) extraocular muscles. Electromyographic studies of

DRS record co-contraction of the LR muscle with the MR muscle, and less frequently co-contraction of the LR muscle with the IR or SR muscles (Huber, 1974; Strachan and Brown, 1972). However, development of aberrant branches of the oculomotor nerve in the absence of the abducens nerve have not been documented *in vivo*, and it is unknown whether aberrant branching results from primary pathology in the oculomotor nerve, or is secondary to abducens nerve hypoplasia.

In this study, we report *MAFB* as a human DRS disease gene. The mouse *Mafb* (*Krml1*) encodes a transcription factor of the basic leucine zipper family (Kataoka et al., 1994). Previous studies have shown that *Mafb* is required for proper hindbrain segmentation (Cordes and Barsh, 1994; McKay et al., 1994), and regulates other transcription factors involved in hindbrain patterning, such as *Hoxb3* (Manzanares et al., 1997). *Mafb* has also been shown to play a role in the differentiation of kidney podocytes (Sadl et al., 2002), macrophages (Aziz et al., 2009), and α and β cells in the pancreas (Artner et al., 2007; Artner et al., 2006). Here, we identify haploinsufficient and dominant negative *MAFB* mutations in patients with DRS and propose a threshold model for loss of MAFB function causing DRS and inner ear defects. We also demonstrate that *Mafb* knockout mice recapitulate human DRS pathology and can serve as an effective disease model, and establish that the aberrant branching of the oculomotor nerve in DRS is secondary to disruptions of abducens nerve development. Our findings broaden our understanding of the etiology of DRS and suggest that both environmental and genetic insults to the abducens nerve in early development can cause this disorder.

RESULTS

Mutations in *MAFB* cause Duane retraction syndrome

We enrolled a dominant pedigree FA that segregated DRS together with congenital hearing loss (**Figure 1A**). Affected members of this pedigree had clinical DRS and inner ear common cavity anomalies (**Figure 1B** and **1C**). The inner ear defects suggested a disruption of hindbrain development similar to that previously reported in patients with *HOXA1* mutations and in $Mafb^{KO/KO}$ mice (Tischfield et al., 2005; Yu et al., 2013). Affected members did not harbor a *HOXA1* mutation. Thus, we screened *MAFB* in this pedigree and in an additional 410 DRS probands for exonic mutations and copy number variations (**Table S1**). We identified heterozygous single base pair frameshift deletions in pedigrees FA (*de novo*), 0819 (*de novo*), and PM, and a heterozygous full gene deletion in pedigree N (**Figure 1A**, **Figure S1** and **S2**). These *MAFB* variants were not present in the ExAC database. Notably, the affected individual in pedigree 0819 represents the first reported case of a *de novo* genetic mutation causing non-syndromic simplex DRS. Unlike pedigree FA, affected members of pedigrees 0819, PM, and N had isolated DRS with no hearing loss (**Table 1**).

***Mafb* knockout mice model DRS pathogenesis**

To examine whether loss of MAFB causes DRS *in vivo*, we studied *Mafb* knockout mice (Yu et al., 2013). At E11.5, $Mafb^{WT/WT}$ embryos have normal hindbrain and cranial nerve development (**Figure 2A** and **2D**). $Mafb^{WT/KO}$ embryos have hypoplastic abducens nerves but otherwise normal hindbrain development (**Figure 2B** and **2E**). $Mafb^{KO/KO}$ embryos do not develop rhombomeres 5 and 6, and as a result have absent abducens nerves and fusion of the glossopharyngeal and vagus nerves (**Figure 2C** and **2F**).

Using a novel orbital dissection technique, we observe that by E12.5, in *Mafb*$^{WT/WT}$ embryos the abducens nerve is present in the orbit and contacts the developing LR muscle (**Figure 2G**). In *Mafb*$^{WT/KO}$ embryos a hypoplastic abducens nerve contacts the developing LR muscle, but an aberrant branch of the oculomotor nerve also begins to form in the direction of the LR muscle (**Figure 2H**). In *Mafb*$^{KO/KO}$ embryos the abducens nerve is absent, and an aberrant branch of the oculomotor nerve forms and contacts the developing LR muscle along a similar trajectory as the wildtype abducens nerve (**Figure 2I**). This aberrant branch arises from a decision region of the oculomotor nerve where it branches into its superior and inferior divisions (Cheng et al., 2014).

By E13.5, in *Mafb*$^{WT/WT}$ embryos the abducens nerve and LR muscle continue to develop, and there are no aberrant branches from the oculomotor nerve (**Figure 2J**). In *Mafb*$^{WT/KO}$ embryos the hypoplastic abducens nerve remains in contact with the LR muscle, and the oculomotor nerve forms additional aberrant branches towards the LR muscle (**Figure 2K**). In *Mafb*$^{KO/KO}$ embryos the oculomotor nerve forms a clear distal aberrant branch in addition to the more proximal aberrant branch that formed at E12.5 (**Figure 2L**). The distal aberrant branch arises from a second decision region where the oculomotor inferior division further branches to innervate the MR muscle, the inferior rectus (IR) muscle, and the inferior oblique (IO) muscle.

By E16.5, *Mafb*$^{WT/WT}$ embryos have developed the adult configuration of the extraocular muscles (EOMs) and cranial nerves in the orbit (**Figure 2M**). In *Mafb*$^{WT/KO}$ embryos the abducens nerve remains hypoplastic, and the LR muscle is innervated both by the hypoplastic abducens nerve and by aberrant branches of the oculomotor nerve (**Figure 2N**). In *Mafb*$^{KO/KO}$ embryos the LR muscle is innervated by two distinct aberrant branches of the oculomotor nerve, one smaller

proximal branch and one larger distal branch (**Figure 2O**). The distal aberrant branch always appears larger than the proximal aberrant branch.

Our findings confirm human DRS pathology in an animal model for the first time, and broaden our understanding of the developmental mechanism of DRS. Knocking out *Mafb* selectively disrupts abducens nerve development, since *Mafb* expression is restricted to rhombomeres 5 and 6 in early development and is not found in developing midbrain oculomotor neurons (Cordes and Barsh, 1994; Giudicelli et al., 2003; Kim et al., 2005; Sadl et al., 2003). Thus, the DRS pathology in *Mafb* knockout mice demonstrates that the aberrant innervation of the LR muscle by the oculomotor nerve arises secondarily to absent or reduced LR muscle innervation by the abducens nerve.

Loss of MAFB function below a 50% threshold causes both DRS and inner ear defects

We next investigated whether DRS and hearing loss in pedigree FA shared a common etiology. We hypothesized that pedigree FA harbored a heterozygous dominant-negative *MAFB* mutation that caused the more severe phenotype of DRS with hearing loss, while the pedigrees with isolated DRS had heterozygous loss-of-function mutations that resulted in haploinsufficiency. This hypothesis was supported by *HOXA1* patients who have both DRS and inner ear defects (Tischfield et al., 2005), and by $Mafb^{KO/KO}$ mice, which also have inner ear defects (Yu et al., 2013). Further support comes from *kreisler* (*kr*) mice (Hertwig, 1942), which harbor a hypomorphic *Mafb* allele (Eichmann et al., 1997; Sing et al., 2009). *kr*/+ mice have normal inner ear and abducens nerve development, while *kr*/*kr* mice have inner ear defects and absent abducens nerves (Cordes and Barsh, 1994; Deol, 1964; McKay et al., 1994). Although we have

not examined the orbits of *kr/kr* mice, our findings in *Mafb*$^{KO/KO}$ mice demonstrate that absence of the abducens nerves in early development is sufficient to cause DRS.

MAFB consists of three critical functional domains: an extended homology region (EHR) and a basic region (BR) required for DNA binding, and a leucine zipper (LZ) required for dimerization (Kataoka et al., 1994; Kerppola and Curran, 1994). The frameshift mutation in pedigree 0819 occurs between the N-terminal polyhistidine regions, and is predicted to result in a mutant MAFB protein that retains the first polyhistidine region followed by 77 altered amino acids, truncating at a new stop codon (**Figure 3A**). The frameshift mutation in pedigree PM occurs at the beginning of the EHR, and is predicted to result in a mutant MAFB protein that retains the first 3 amino acids of the EHR followed by 8 altered amino acids, truncating at the same stop codon as the 0819-mutant MAFB (**Figure 3A**). Since the 0819-mutant MAFB protein lacks the EHR, BR, and LZ domains, and the PM-mutant MAFB protein lacks most of the EHR and all of the BR and LZ domains, both are predicted to have no dimerization or DNA binding function. In contrast, the frameshift mutation in pedigree FA occurs in the LZ, and is predicted to result in a mutant MAFB protein that retains the EHR, BR, and first leucine of the LZ, followed by 125 altered amino acids (**Figure 3A**).

We found expression of the FA-mutant *MAFB* mRNA in FA patient-derived lymphoblasts (**Table S2**, **Figure S3**), and we successfully overexpressed FA- and 0819-mutant *MAFB* constructs *in vitro* (**Figure S4**). We measured the transcriptional activity of wildtype, FA-mutant, and 0819-mutant MAFB proteins by luciferase assay and found no activity in either mutant alone (**Figure 3B**). We then co-expressed wildtype MAFB with each mutant and found that FA-mutant MAFB, but not 0819-mutant MAFB, reduced the transcriptional activity of the wildtype protein

(**Figure 3B**). These data support a heterozygous dominant-negative mechanism for FA-mutant MAFB, and a heterozygous loss-of-function mechanism for 0819-mutant MAFB.

Combining our human and mouse data, we propose a threshold model for variable loss of MAFB function (**Figure 3C**). The heterozygous loss-of-function *MAFB* alleles in pedigrees N, 0819, and PM result in 50% protein function and cause isolated DRS, consistent with DRS pathology and normal inner ear development in $Mafb^{WT/KO}$ mice. The heterozygous dominant-negative *MAFB* allele in pedigree FA results in less than 50% protein function and causes both DRS and inner ear defects, consistent with absent abducens nerves and inner ear defects in *kr/kr* mice, as well as DRS pathology and inner ear defects in $Mafb^{KO/KO}$ mice.

DISCUSSION

In this study, we use a combination of human genetics and an instructive mouse model to further our understanding of DRS. We establish *MAFB* as a new DRS disease gene, and find that *MAFB* mutations are present in ~1% of DRS probands in our cohort, making it a more common cause of DRS than the previously identified disease genes *CHN1* (Miyake et al., 2008), *HOXA1* (Tischfield et al., 2005), or *SALL4* (Al-Baradie et al., 2002). We propose that loss of MAFB function in developing hindbrain below a 50% threshold can cause both DRS and inner ear defects. We support this hypothesis through genotype-phenotype correlations in human and mouse. There are previous reports of heterozygous dominant-negative mutations causing more severe human phenotypes than heterozygous loss-of-function mutations in the same gene (Antony-Debre et al., 2015; Kondo et al., 2002), but to our knowledge *MAFB* is the first such

case to be supported by an animal model. Our findings also represent the first loss-of-function human mutations reported in *MAFB* and demonstrate allelic diversity in this gene (Walsh and Engle, 2010). Hotspot missense mutations in the N-terminal transactivation domain were previously reported to cause multicentric carpotarsal osteolysis (Zankl et al., 2012), and mutations in noncoding regions have been associated with cleft lip/cleft palate (Beaty et al., 2010). These previously reported mutations are most likely gain-of-function since they do not disrupt the EHR, BR, or LZ domains of MAFB, and therefore act through different pathogenic mechanisms than the loss-of-function and dominant-negative mutations we have identified.

Although DRS was first described clinically in 1905 (Duane, 1996), the developmental etiology remained unclear due to the lack of animal models that could reveal the underlying developmental neuropathology. Our work in *Mafb* knockout mice confirms human DRS pathology and demonstrates DRS pathogenesis in an animal model for the first time. Using a novel dissection technique to visualize the cranial nerves and extraocular muscles in the embryo, we elucidate the developmental mechanism of DRS by selectively disrupting the abducens nerve and observing secondary aberrant branching of the oculomotor nerve to innervate the LR muscle. This provides the first experimental evidence to link proposed environmental causes of DRS, such as ischemia or teratogens (Parsa and Robert, 2013), with established genetic causes of DRS, such as hindbrain malformations in *HOXA1* patients (Tischfield et al., 2005), into one common etiology. This has important clinical implications, since it suggests that both environmental and genetic disruptions of the abducens nerve in embryonic development can result in secondary aberrant innervation of the LR muscle by the oculomotor nerve, and offers insight into why DRS is far more common than a congenital sixth nerve palsy in the absence of globe retraction.

The aberrant branching of the *Mafb*$^{KO/KO}$ oculomotor nerve at its two decision regions (Cheng et al., 2014) may explain the highly stereotypic co-contraction of the LR with the MR muscle, and the less frequent co-contraction of the LR with the SR and IR muscles, as reported by human electromyography (Huber, 1974; Strachan and Brown, 1972). At its proximal decision region, the oculomotor nerve divides into superior and inferior divisions, and axons destined for any of its innervated muscles could be rerouted to form the small branches that aberrantly innervate the LR muscle in DRS. In particular, misdirection of a subset of superior division axons normally destined for the SR would account for the rare reports of LR and SR muscle co-contraction. In *Mafb*$^{KO/KO}$ embryos, we find that most of the aberrant innervation arises from the distal decision region; here, axon rerouting could account for co-contraction of the LR with the MR or IR muscles. Thus, it is likely that the extraocular muscles ensure their innervation in early development by secreting shared guidance factors that induce nerve branching and directional growth along defined trajectories, and that the oculomotor and abducens axons compete for these guidance factors at two specific decision regions. Moreover, because co-contraction of the LR with the MR muscle is the most common aberrant innervation pattern in DRS, extraocular muscle innervation may also be programmed along the horizontal and vertical axes.

EXPERIMENTAL PROCEDURES

Clinical data and mutation screening. See Supplemental Experimental Procedures.

***Mafb* knockout mice.** *Mafb*$^{WT/flox}$ mice were generously provided from the laboratory of Lisa Goodrich at Harvard Medical School (Yu et al., 2013). These were crossed to ubiquitously

expressing *EIIa-Cre* mice to knock out *Mafb*. *Mafb*$^{WT/KO}$ mice were used for heterozygous crosses. *Mafb*$^{KO/KO}$ mice were used for embryonic studies but die at birth due to respiratory defects (Blanchi et al., 2003). DNA was extracted from embryonic yolk sac using Extracta DNA Prep (Quanta Biosciences) and used for genotyping with *Mafb* wildtype and KO primers (**Table S2**). *ISL*^{MN}*:GFP* reporter mice were generously provided from the laboratory of Samuel Pfaff at The Salk Institute (Lewcock et al., 2007), and crossed to *Mafb* mice to allow for visualization of motor axons in development.

Orbital dissections. E12.5 and E16.5 *Mafb-ISL*^{MN}*:GFP* mouse embryos were fixed overnight in 4% PFA. Brain tissue around the orbit was dissected away, leaving the distal cranial nerves and EOMs intact. The orbits were incubated with anti-actin α-smooth muscle-Cy3 antibody (Sigma-Aldrich) for 3 days at 4°C. After washing x3 with PBS, the orbits were further dissected and flat mounted in 70% glycerol and 1% 1M KOH in PBS. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope and Zen software (Zeiss). Images were processed with Imaris software (Bitplane). $N \geq 3$ were used for each experiment.

Whole mount immunohistochemistry. E11.5 *Mafb-ISL*^{MN}*:GFP* mouse embryos were prepared as described previously (Huber et al., 2005). Embryos were stained with mouse anti-neurofilament (clone 2H3, Developmental Studies Hybridoma Bank) at 1:500 and GFP tag antibody Alexa Fluor 488 conjugate (Thermo Fisher Scientific) at 1:500 as primaries, and goat anti-mouse Alexa Fluor 594 conjugate (Thermo Fisher Scientific) at 1:1000 as a secondary. Embryos were cleared with BABB and placed into a custom designed holding chamber for confocal imaging. $N \geq 3$ were used for each experiment.

Cell culture, transfection, and DNA expression. See Supplemental Experimental Procedures.

Western blot and Luciferase assay. See Supplemental Experimental Procedures.

Statistical analyses. Data were analyzed in GraphPad Prism. One-way ANOVA was used to measure differences in relative fold activation. Error bars report standard error of the mean.

AUTHOR CONTRIBUTIONS

J.G.P., M.A.T., A.A.N., and E.C.E. designed the study. J.G.P., M.A.T., S.A.D.G., and W.-M.C. performed genetics experiments. J.G.P. and A.A.N. performed mouse experiments. J.G.P., L.C., and S.A.D.G. performed *in vitro* experiments. G.M., T.M.B., C.G.S., D.G.H., and I.G. referred and examined patients. C.D.R. read CT scans. J.G.P. prepared the figures. J.G.P. and E.C.E. wrote the manuscript.

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REFERENCES

Al-Baradie, R., Yamada, K., St Hilaire, C., Chan, W.M., Andrews, C., McIntosh, N., Nakano, M., Martonyi, E.J., Raymond, W.R., Okumura, S., *et al.* (2002). Duane radial ray syndrome (Okihiro syndrome) maps to 20q13 and results from mutations in SALL4, a new member of the SAL family. Am J Hum Genet *71*, 1195-1199.

Antony-Debre, I., Manchev, V.T., Balayn, N., Bluteau, D., Tomowiak, C., Legrand, C., Langlois, T., Bawa, O., Tosca, L., Tachdjian, G., *et al.* (2015). Level of RUNX1 activity is critical for leukemic predisposition but not for thrombocytopenia. Blood *125*, 930-940.

Artner, I., Blanchi, B., Raum, J.C., Guo, M., Kaneko, T., Cordes, S., Sieweke, M., and Stein, R. (2007). MafB is required for islet beta cell maturation. Proceedings of the National Academy of Sciences of the United States of America *104*, 3853-3858.

Artner, I., Le Lay, J., Hang, Y., Elghazi, L., Schisler, J.C., Henderson, E., Sosa-Pineda, B., and Stein, R. (2006). MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. Diabetes *55*, 297-304.

Aziz, A., Soucie, E., Sarrazin, S., and Sieweke, M.H. (2009). MafB/c-Maf deficiency enables self-renewal of differentiated functional macrophages. Science *326*, 867-871.

Beaty, T.H., Murray, J.C., Marazita, M.L., Munger, R.G., Ruczinski, I., Hetmanski, J.B., Liang, K.Y., Wu, T., Murray, T., Fallin, M.D., *et al.* (2010). A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. Nature genetics *42*, 525-529.

Blanchi, B., Kelly, L.M., Viemari, J.C., Lafon, I., Burnet, H., Bevengut, M., Tillmanns, S., Daniel, L., Graf, T., Hilaire, G., *et al.* (2003). MafB deficiency causes defective respiratory rhythmogenesis and fatal central apnea at birth. Nature neuroscience *6*, 1091-1100.

Cheng, L., Desai, J., Miranda, C.J., Duncan, J.S., Qiu, W., Nugent, A.A., Kolpak, A.L., Wu, C.C., Drokhlyansky, E., Delisle, M.M., *et al.* (2014). Human CFEOM1 mutations attenuate KIF21A autoinhibition and cause oculomotor axon stalling. Neuron *82*, 334-349.

Cordes, S.P., and Barsh, G.S. (1994). The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor. Cell *79*, 1025-1034.

Demer, J.L., Clark, R.A., Lim, K.H., and Engle, E.C. (2007). Magnetic resonance imaging of innervational and extraocular muscle abnormalities in Duane-radial ray syndrome. Investigative ophthalmology & visual science *48*, 5505-5511.

Deol, M.S. (1964). The Abnormalities of the Inner Ear in Kreisler Mice. Journal of embryology and experimental morphology *12*, 475-490.

Duane, A. (1996). Congenital deficiency of abduction, associated with impairment of adduction, retraction movements, contraction of the palpebral fissure and oblique movements of the eye. 1905. Archives of ophthalmology *114*, 1255-1256; discussion 1257.

Eichmann, A., Grapin-Botton, A., Kelly, L., Graf, T., Le Douarin, N.M., and Sieweke, M. (1997). The expression pattern of the mafB/kr gene in birds and mice reveals that the kreisler phenotype does not represent a null mutant. Mechanisms of development *65*, 111-122.

Engle, E.C. (2010). Human genetic disorders of axon guidance. Cold Spring Harbor perspectives in biology *2*, a001784.

Giudicelli, F., Gilardi-Hebenstreit, P., Mechta-Grigoriou, F., Poquet, C., and Charnay, P. (2003). Novel activities of Mafb underlie its dual role in hindbrain segmentation and regional specification. Developmental biology *253*, 150-162.

Hertwig, P. (1942). Neue Mutationen und Koppellungsgruppen bei der Hausmaus. Z indukt Abstamm VerebLehre *80*, 220-246.

Hotchkiss, M.G., Miller, N.R., Clark, A.W., and Green, W.R. (1980). Bilateral Duane's retraction syndrome. A clinical-pathologic case report. Archives of ophthalmology *98*, 870-874.

Huber, A. (1974). Electrophysiology of the retraction syndromes. The British journal of ophthalmology *58*, 293-300.

Huber, A.B., Kania, A., Tran, T.S., Gu, C., De Marco Garcia, N., Lieberam, I., Johnson, D., Jessell, T.M., Ginty, D.D., and Kolodkin, A.L. (2005). Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. Neuron *48*, 949-964.

Kataoka, K., Fujiwara, K.T., Noda, M., and Nishizawa, M. (1994). MafB, a new Maf family transcription activator that can associate with Maf and Fos but not with Jun. Molecular and cellular biology *14*, 7581-7591.

Kerppola, T.K., and Curran, T. (1994). A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. Oncogene *9*, 3149-3158.

Kim, F.A., Sing l, A., Kaneko, T., Bieman, M., Stallwood, N., Sadl, V.S., and Cordes, S.P. (2005). The vHNF1 homeodomain protein establishes early rhombomere identity by direct regulation of Kreisler expression. Mechanisms of development *122*, 1300-1309.

Kondo, S., Schutte, B.C., Richardson, R.J., Bjork, B.C., Knight, A.S., Watanabe, Y., Howard, E., de Lima, R.L., Daack-Hirsch, S., Sander, A., *et al.* (2002). Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nature genetics *32*, 285-289.

Lewcock, J.W., Genoud, N., Lettieri, K., and Pfaff, S.L. (2007). The ubiquitin ligase Phr1 regulates axon outgrowth through modulation of microtubule dynamics. Neuron *56*, 604-620.

Manzanares, M., Cordes, S., Kwan, C.T., Sham, M.H., Barsh, G.S., and Krumlauf, R. (1997). Segmental regulation of Hoxb-3 by kreisler. Nature *387*, 191-195.

McKay, I.J., Muchamore, I., Krumlauf, R., Maden, M., Lumsden, A., and Lewis, J. (1994). The kreisler mouse: a hindbrain segmentation mutant that lacks two rhombomeres. Development *120*, 2199-2211.

Miller, N.R., Kiel, S.M., Green, W.R., and Clark, A.W. (1982). Unilateral Duane's retraction syndrome (Type 1). Archives of ophthalmology *100*, 1468-1472.

Miyake, N., Chilton, J., Psatha, M., Cheng, L., Andrews, C., Chan, W.M., Law, K., Crosier, M., Lindsay, S., Cheung, M., *et al.* (2008). Human CHN1 mutations hyperactivate alpha2-chimaerin and cause Duane's retraction syndrome. Science *321*, 839-843.

Parsa, C.F., and Robert, M.P. (2013). Thromboembolism and congenital malformations: from Duane syndrome to thalidomide embryopathy. JAMA ophthalmology *131*, 439-447.

Sadl, V., Jin, F., Yu, J., Cui, S., Holmyard, D., Quaggin, S., Barsh, G., and Cordes, S. (2002). The mouse Kreisler (Krml1/MafB) segmentation gene is required for differentiation of glomerular visceral epithelial cells. Developmental biology *249*, 16-29.

Sadl, V.S., Sing, A., Mar, L., Jin, F., and Cordes, S.P. (2003). Analysis of hindbrain patterning defects caused by the kreisler(enu) mutation reveals multiple roles of Kreisler in hindbrain segmentation. Developmental dynamics : an official publication of the American Association of Anatomists *227*, 134-142.

Sing, A., Pannell, D., Karaiskakis, A., Sturgeon, K., Djabali, M., Ellis, J., Lipshitz, H.D., and Cordes, S.P. (2009). A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. Cell *138*, 885-897.

Strachan, I.M., and Brown, B.H. (1972). Electromyography of extraocular muscles in Duane's syndrome. The British journal of ophthalmology *56*, 594-599.

Tischfield, M.A., Bosley, T.M., Salih, M.A., Alorainy, I.A., Sener, E.C., Nester, M.J., Oystreck, D.T., Chan, W.M., Andrews, C., Erickson, R.P., *et al.* (2005). Homozygous HOXA1 mutations disrupt human brainstem, inner ear, cardiovascular and cognitive development. Nature genetics *37*, 1035-1037.

Walsh, C.A., and Engle, E.C. (2010). Allelic diversity in human developmental neurogenetics: insights into biology and disease. Neuron *68*, 245-253.

Yu, W.M., Appler, J.M., Kim, Y.H., Nishitani, A.M., Holt, J.R., and Goodrich, L.V. (2013). A Gata3-Mafb transcriptional network directs post-synaptic differentiation in synapses specialized for hearing. eLife *2*, e01341.

Zankl, A., Duncan, E.L., Leo, P.J., Clark, G.R., Glazov, E.A., Addor, M.C., Herlin, T., Kim, C.A., Leheup, B.P., McGill, J., *et al.* (2012). Multicentric carpotarsal osteolysis is caused by mutations clustering in the amino-terminal transcriptional activation domain of MAFB. Am J Hum Genet *90*, 494-501.

FIGURE LEGENDS

Figure 1. Mutations in *MAFB* cause DRS. (**A**) Pedigrees FA, N, 0819, and PM segregate mutations in *MAFB* as noted. Three of the four affected FA pedigree members also have unilateral or bilateral congenital hearing loss (indicated by *). (**B**) FA IV:1 has bilateral DRS, characterized by bilaterally limited eye abduction and narrowing of the palpebral fissures with globe retraction during attempted eye adduction. (**C**) Axial CT images of the right temporal bone of a healthy control with normal cochlea and vestibule (arrows), and of individual FA IV:1 who has a cystic common cavity anomaly (arrow).

Figure 2. *Mafb* knockout mice embryos demonstrate DRS pathology. (**A-F**) Whole mount sagittal confocal images at E11.5. (**A**) $Mafb^{WT/WT}$ embryos have normal hindbrain cranial nerve development. White line indicates region of developing rhombomeres 5 and 6. (**B**) $Mafb^{WT/KO}$ embryos have abducens hypoplasia but no other major abnormalities of hindbrain cranial nerve development. (**C**) $Mafb^{KO/KO}$ embryos have loss of rhombomeres 5 and 6 resulting in loss of hindbrain area (white line), absent abducens nerve, and fusion of the glossopharyngeal nerve with the vagus nerve (arrow). (**D-F**) Medial sagittal sections highlighting the developing oculomotor and abducens nerves. (**D**) In $Mafb^{WT/WT}$ embryos, the abducens nerve is present (short arrow) and reaches the developing eye. (**E**) $Mafb^{WT/KO}$ embryos have a hypoplastic abducens nerve (short arrow). (**F**) $Mafb^{KO/KO}$ embryos have an absent abducens nerve (short arrow). (**G-O**) Confocal images of the right orbit from the inferior view. (**G**) At E12.5 in $Mafb^{WT/WT}$ embryos, the abducens nerve (arrowhead) innervates the LR muscle, while the oculomotor nerve innervates the IR muscle and developing IO muscle and does not send any axons towards the LR muscle. (**H**) In $Mafb^{WT/KO}$ embryos, the abducens nerve is hypoplastic (arrowhead) and innervates the LR muscle, while the oculomotor nerve begins to send aberrant branches towards the LR muscle (white arrow). (**I**) In $Mafb^{KO/KO}$ embryos, the abducens nerve is absent (arrowhead), and the oculomotor nerve sends many aberrant branches towards the LR muscle (arrow). (**J**) At E13.5 in $Mafb^{WT/WT}$ embryos, the abducens nerve (arrowhead), oculomotor nerve, and EOMs continue to develop normally. (**K**) In $Mafb^{WT/KO}$ embryos, the hypoplastic abducens nerve (arrowhead) innervates the LR muscle, while the oculomotor nerve forms distal aberrant branches towards the LR muscle (yellow arrow) in addition to the proximal aberrant branch formed earlier (white arrow). (**L**) In $Mafb^{KO/KO}$ embryos, the abducens nerve is absent

(arrowhead), and the oculomotor nerve sends a distinct distal aberrant branch towards the LR muscle (yellow arrow) in addition to the proximal aberrant branch formed earlier (white arrow). (**M**) *Mafb*$^{WT/WT}$ embryos at E16.5 have the final developmental pattern of the orbit, with the abducens nerve (arrowhead) innervating the LR muscle. (**N**) In *Mafb*$^{WT/KO}$ embryos the abducens nerve remains hypoplastic (arrowhead), and a proximal aberrant branch (white arrow) and a distal aberrant branch (yellow arrow) of the oculomotor nerve also innervate the LR muscle. (**O**) In *Mafb*$^{KO/KO}$ embryos the abducens nerve remains absent (arrowhead), and a proximal aberrant branch (white arrow) and a distal aberrant branch (yellow arrow) of the oculomotor nerve innervate the LR muscle instead. The distal aberrant branch always appears larger than the proximal aberrant branch. III, oculomotor nerve; IV, trochlear nerve; V, trigeminal nerve; VI, abducens nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagus nerve; IO, inferior oblique; IR, inferior rectus; LR, lateral rectus; scale bar = 100 μm.

Figure 3. Less than 50% MAFB function causes DRS and inner ear defects. (**A**) N has a full gene deletion and therefore no mutant MAFB. 0819 and PM are predicted to have truncated MAFB proteins that lack the EHR, BR, and LZ domains followed by 77 and 8 altered amino acids, respectively. FA is predicted to have a MAFB protein that retains the wildtype EHR, BR, and beginning of the LZ, followed by 125 altered amino acids. (**B**) Luciferase assay shows wildtype MAFB increases transcription by approximately 150 fold. The 0819- or FA-mutant protein alone does not have any transcriptional activity. Co-expression of wildtype MAFB with FA-mutant, but not 0819-mutant MAFB reduces the transcriptional activity of the wildtype protein compared to wildtype alone. **, $p<0.01$ by one-way ANOVA. (**C**) At greater than 50% MAFB function, *Mafb*$^{WT/WT}$ and *kr*/+ mice have no phenotype. At 50% MAFB function,

Mafb$^{WT/KO}$ mice and members of pedigrees N, 0819, and PM with heterozygous loss-of-function mutations have isolated DRS. At less than 50% MAFB function, *kr/kr* mice, members of pedigree FA with a dominant-negative mutation, and *Mafb*$^{KO/KO}$ mice have both DRS and inner ear defects.

TABLES

Individual	Mutation	DRS	Hearing Loss
N II:2	*MAFB* deletion	Yes Bilateral	No
N II:5	*MAFB* deletion	Yes	No
N II:6	*MAFB* deletion	Yes	No
N III:3	*MAFB* deletion	Yes Bilateral	No
N III:5	*MAFB* deletion	Yes Unilateral (right side)	No
N III:6	*MAFB* deletion	Yes Bilateral	No
FA II:2	c.802delA	Yes Unilateral (right side)	Yes Unilateral (right side)
FA III:1	c.802delA	Yes Unilateral (right side)	No
FA III:3	c.802delA	Yes Unilateral (right side)	Yes Unilateral (right side)
FA IV:1	c.802delA	Yes Bilateral	Yes Bilateral
0819 II:2	c.439delG	Yes Bilateral	No
PM II:4	c.644delA	Yes Bilateral	
PM III:5	c.644delA	Yes Bilateral	
PM III:6	c.644delA	Yes Bilateral	

Table 1. Clinical summary of individuals with mutations in *MAFB*. Affected members of pedigrees N, 0819, and PM have isolated DRS. All of the affected members of pedigree FA have DRS, and 3 of 4 also have hearing loss. Notably, individuals with unilateral hearing loss have unilateral DRS on the same side.