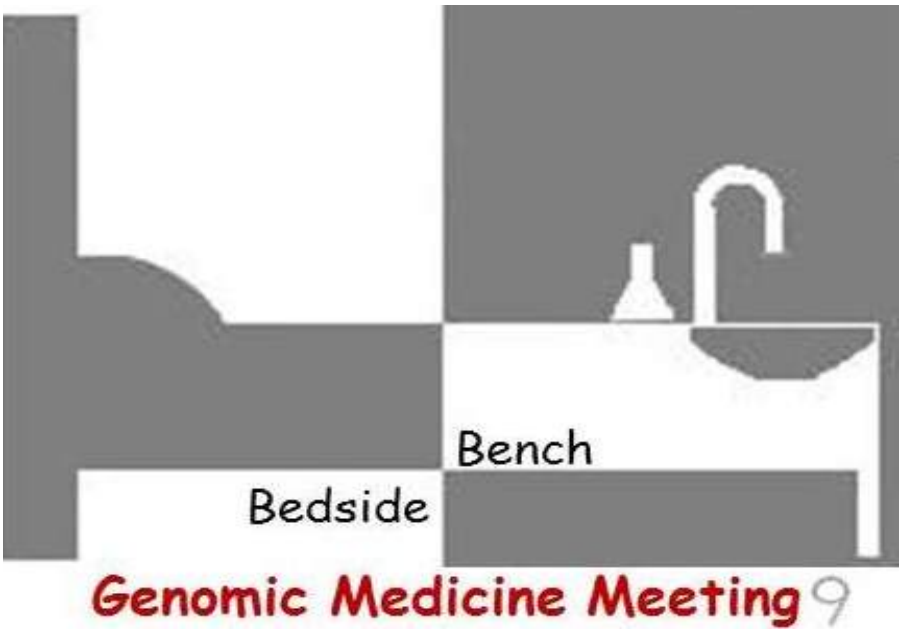

Genomic Medicine Meeting IX

Sheraton Silver Spring
April 19-20, 2016

Genomic Medicine IX:
NHGRI's Genomic Medicine Portfolio

April 19-20, 2016

Sheraton Silver Spring
8777 Georgia Avenue
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Genomic Medicine IX:
NHGRI's Genomic Medicine Portfolio

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Sheraton Silver Spring
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FOCUS of GENOMIC MEDICINE IX MEETING

Bedside to Bench – Mind the Gaps

GM9 will focus on facilitating bedside back to bench research by focusing on one of the most vexing problems in clinical sequencing: characterizing and interpreting variants of uncertain significance (VUS). Objectives include to:

1. Review examples of successful interactions between basic scientists and clinical genomicists and explore what made them successful;
2. Identify ways to enhance interactions between basic scientists and clinical genomicists (aka, the virtuous cycle of bench to bedside and back again)
3. Determine how better to integrate basic science research efforts with clinically important questions, to enhance the exploration of clinical implications of basic discoveries.

Topic areas to be covered:

- How basic science lends insights into disease mechanisms to facilitate effective approaches for understanding the function of VUS and relevance to disease mechanisms.
- Computational and informatics approaches to prediction and annotation of genomic variant function.
- Efforts and strategies for data integration through the development and implementation of biomedical ontologies.
- Facilitating bedside-back-to-bench research.

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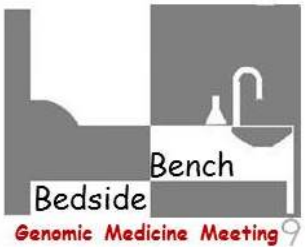

Sheraton Silver Spring
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Tuesday, April 19th

LOCATION: **Sheraton Silver Spring – Magnolia Ballroom**
WEBCAST: **Genome TV - *http://www.genome.gov/GenomeTV/***
TWITTER: **#GenomicMed9**

7:30 AM	Registration and Breakfast	Registration Table
	Session 1: Introduction, Background ***Dan Roden, Moderator***	
8:30 AM	Welcome, Introductions, and Goals of the Meeting	Carol Bult Teri Manolio Eric Green
8:50 AM	Convincing Clinicians to Use Functionalized Genomic Information	Howard Jacob
9:05 AM	Magnitude of the Problem – Basic Science Perspective On Need for Integration	Monte Westerfield
9:30 AM	Magnitude of the Problem – Clinical Perspective on Need For Integration	Gail Herman
9:55 AM	Discussion	
10:40 AM	Break	
	Session 2: Vexing Clinical Problems Needing Basic Input ***Laura Rodriguez, Moderator***	
11:00 AM	Speeding Functional Assessment to Benefit Patients	Stephen Kingsmore
11:20 AM	*De Novo* Variants that Inform Clinical Phenotypes	Christine Seidman
11:40 AM	Discussion	

Genomic Medicine IX:
NHGRI's Genomic Medicine Portfolio

April 19-20, 2016

Tuesday, April 19th…continued

12:25 PM	LUNCH	
	Session 3: From Variant to Disease Mechanisms – Specific Examples of How Model Systems have used Genetic / Genomic Approaches to Lend Insight into Human Disease that had Clinical Relevance ***Howard Jacob, Moderator***	
1:30 PM	Integrating Model Organism Data around Clinical Genomics	Calum MacRae
1:50 PM	Leveraging Congenital Heart Disease Mouse Model Findings to Improve Clinical Outcome	Cecilia Lo
2:10 PM	Discussion	
3:00 PM	BREAK	
	Session 4: Computational Approaches to Variant Function Prediction Methods for Predicting Functional Consequences of Variants ***Marc Williams, Moderator***	
3:20 PM	Leveraging Massive-Scale Databases of Human Genetic Variation	Daniel MacArthur
3:40 PM	Empowering Variant Effect Prediction with Large Scale Mutagenesis Data	Douglas Fowler
4:00 PM	Discussion	
4:45 PM	***PANEL 1: Topics to be Defined Based on Day 1 Sessions*** ***Teri Manolio, Moderator*** ***Les Biesecker, Greg Cooper, Cat Lutz, Erin Ramos, and Liz Worthey***	
5:45 PM	First Day Summary and Discussion	Carol Bult Teri Manolio
6:00 PM	ADJOURN	

On Your Own for Dinner (see map in registration materials)

Genomic Medicine IX:
NHGRI's Genomic Medicine Portfolio

Wednesday, April 20th

7:30 AM	Breakfast	
	Session 5: Functionalizing VUS's ***Howard McLeod, Moderator***	
8:30 AM	Massively Parallel Functional Analysis of Missense Mutations In BRCA1 for Interpreting Variants of Uncertain Significance	Lea Starita
8:50 AM	CRISPR-Cas9 Mediated Mouse Model Creation and Transcription Regulation	Haoyi Wang
9:10 AM	Discussion	
9:55 AM	BREAK	
	Session 6: Biomedical Phenotype Ontologies and Data Integration ***Carol Bult, Moderator***	
10:15 AM	Translating Human to Models and Back Again: Phenotype Ontologies for Data Integration and Discovery	Melissa Haendel
10:35 AM	Data Integration: Genome X Transcriptome X EMR	Nancy Cox
10:55 AM	Discussion	
11:35 AM	Providing Clinical Utility: Who Is the Judge and Jury? Webex link: https://dukemed.webex.com/dukemed/j.php?MTID=m3e8636840d17de8e1eaf15051d925752 Meeting number: 738 378 332 Meeting password: GM904202016	Robert Nussbaum
11:50 AM	Discussion	
12:00 PM	WORKING LUNCH	
1:00 PM	***Panel 2: Topics to be Defined Based on Day 2 Sessions*** ***Rex Chisholm, Moderator*** ***Calum MacRae, Jose Ordovas, Peter Robinson, Wendy Rubinstein, Barbara Stranger***	
2:00 PM	Summary Discussion: Promoting Bedside-Back-To-Bench Research ▪ Prioritizing Variants for Functional/Mechanistic Investigation ▪ Incentivizing Demonstrations of Benignity ▪ Engaging Industry	Carol Bult Teri Manolio
3:30 PM	Next Steps	Carol Bult Teri Manolio
4:00 PM	ADJOURN	

Shroom3 contributes to the maintenance of the glomerular filtration barrier integrity

Nan Cher Yeo,[1,2,12] Caitlin C. O'Meara,[1,2,12] Jason A. Bonomo,[3,4] Kerry N. Veth,[5] Ritu Tomar,[6] Michael J. Flister,[1,2] Iain A. Drummond,[6,7] Donald W. Bowden,[4,8] Barry I. Freedman,[4,9] Jozef Lazar,[1,10] Brian A. Link,[5] and Howard J. Jacob[1,2,11]

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Genome-wide association studies (GWAS) identify regions of the genome correlated with disease risk but are restricted in their ability to identify the underlying causative mechanism(s). Thus, GWAS are useful "roadmaps" that require functional analysis to establish the genetic and mechanistic structure of a particular locus. Unfortunately, direct functional testing in humans is limited, demonstrating the need for complementary approaches. Here we used an integrated approach combining zebrafish, rat, and human data to interrogate the function of an established GWAS locus (*SHROOM3*) lacking prior functional support for chronic kidney disease (CKD). Congenic mapping and sequence analysis in rats suggested *Shroom3* was a strong positional candidate gene. Transferring a 6.1-Mb region containing the wild-type *Shroom3* gene significantly improved the kidney glomerular function in FHH (fawn-hooded hypertensive) rat. The wild-type *Shroom3* allele, but not the FHH *Shroom3* allele, rescued glomerular defects induced by knockdown of endogenous *shroom3* in zebrafish, suggesting that the FHH *Shroom3* allele is defective and likely contributes to renal injury in the FHH rat. We also show for the first time that variants disrupting the actin-binding domain of *SHROOM3* may cause podocyte effacement and impairment of the glomerular filtration barrier.

[Supplemental material is available for this article.]

Genome-wide association studies (GWAS) have identified many genetic loci associated with common complex diseases; however, discovering the exact genes remains problematic for several reasons (Manolio et al. 2009). Furthermore, these loci collectively explain only a small percentage of disease heritability, indicating the need to identify alternative sources of "missing" heritability (Manolio et al. 2009; Chatterjee et al. 2013). Genomic infrastructure available for animal models provides a powerful complementary tool to GWAS (Aitman et al. 2008; Geurts et al. 2009; Cox and Church 2011; Lewis and Tomlinson 2012; Atanur et al. 2013; Varshney et al. 2013). For example, mapping quantitative trait loci (QTL) in mammals and identifying natural mutations in syntenic regions of animal disease models have provided functional support for human risk loci in multiple diseases (Aitman et al. 2008). The ability to then genetically manipulate these disease models enables a direct approach to establish causation (Geurts et al. 2009; Cox and Church 2011). Chronic kidney disease (CKD) is a major health issue and is highly heritable (Jha et al. 2013); however, the majority of risk loci causing CKD are uncharacterized and a vast amount of CKD heritability remains to be explained. Here, we leveraged integrated physiological genomics approaches in zebrafish, rat, and human combined with biochemical structural assessment to dissect a highly replicated GWAS locus (*SHROOM3*) and its contribution to renal impairment. Based on our study, we propose that similar approaches can be used to assess many of the 44 other uncharacterized human CKD loci and potentially other loci associated with common diseases (Okada et al. 2012; O'Seaghdha and Fox 2012).

Eight GWAS reported association between intronic *SHROOM3* variants and CKD (Supplemental Table 1); yet, its role in renal function and disease was unknown, and establishing a functional link between *SHROOM3* and renal physiology had been difficult due to early postnatal lethality of *Shroom3* knockout in mice (Hildebrand and Soriano 1999). *Shroom3* encodes an actin-binding protein that regulates morphogenesis of epithelial cells (Hildebrand and Soriano 1999), and it is expressed in the kidney. We note that *Shroom3* is located within a QTL previously found to contribute to albuminuria in the FHH (Fawn-Hooded Hypertensive) rat (Shiozawa et al. 2000). Sequence analysis further identified 13 protein-coding

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variants in the FHH *Shroom3* gene compared to the wild-type allele, implicating it as a potential candidate gene in the FHH rat model of CKD. In the present study, we investigated the role of *Shroom3* in kidney function by generating a congenic rat in which the wild-type *Shroom3* allele from BN (Brown Norway) rat was introgressed onto the FHH genetic background. We then utilized hypomorphic and tissue-specific disruption of *shroom3* in zebrafish to test the effect of the gene and its allelic variants on kidney function. We demonstrate that the variants disrupting the actin-binding domain of *SHROOM3* may cause podocyte effacement and impairment of the glomerular filtration barrier. This study establishes the first functional link for *SHROOM3* and kidney pathophysiology.

Results

Introgression of the BN *Shroom3* gene onto the FHH genetic background improved glomerular function

We generated a congenic rat strain (named FHH.BN14a) in which a BN-derived 14.50- to 21.40-Mbp region of Chromosome 14 encompassing the *Shroom3* gene was introgressed onto the FHH background and compared its renal phenotypes to the parental FHH strain (Supplemental Fig. 1). At 14 wk of age, both homozygous and heterozygous FHH.BN14a congenic animals showed a significantly lower level of albuminuria, a marker of kidney injury, compared to the FHH rats (Fig. 1A). We measured glomerular permeability to albumin (Palb) in isolated glomeruli from all strains as described previously (O'Meara et al. 2012) and found that Palb in both homozygous and heterozygous FHH.BN14a animals were significantly lower than that in the FHH rats (Fig. 1B). Renal histology showed significantly decreased glomerular sclerosis in the congenic animals compared to the FHH rats (Fig. 1C,D). Electron microscopic analyses of the glomerulus revealed fusion of podocyte foot processes in FHH but not in congenic animals at 18 wk of age (Fig. 1E). These data suggest that replacement of this region containing the BN *Shroom3* allele improved glomerular structure and function in the congenic animals.

We identified 13 amino acid variants (12 missense and a single-amino acid duplication) in the FHH *Shroom3* allele compared with BN rat (Fig. 2A). Six of the 13 variants were predicted computationally to be damaging to *Shroom3* function, and several variants localize to conserved functional domains of the

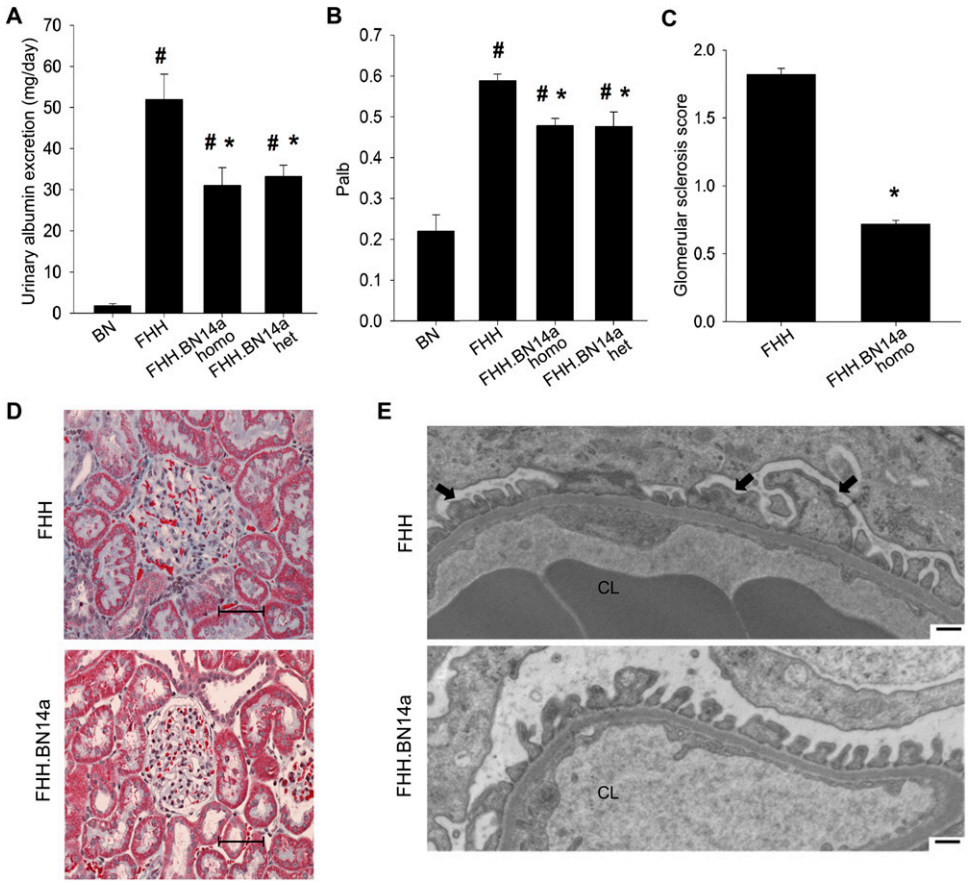

Figure 1. Introgression of the BN *Shroom3* gene onto FHH background improves glomerular and overall kidney function. (*A*) At 14 wk of age, both homozygous and heterozygous FHH.BN14a congenic animals showed a significantly lower degree of albuminuria compared to FHH ($n = 4$, 7, 8, and 3, respectively). (*B*) Both heterozygous and homozygous FHH.BN14a demonstrated significantly improved glomerular permeability (Palb) compared to FHH. ($n = 4$ animals/115 glomeruli, 3 animals/76 glomeruli, and 2 animals/70 glomeruli, respectively. Palb in BN was obtained from previously published data [Rangel-Filho et al. 2005].) (*C*) FHH.BN14a kidney showed a decreased presence of glomerular sclerosis compared to FHH at 14 wk of age. A minimum of 30 glomeruli from three kidneys for each strain were scored for a percentage of sclerosis using a scale from 0 (no sclerosis) to 4 (complete sclerosis). (*D*) Representative trichrome-stained images of glomeruli from FHH and FHH.BN14a are shown. Fibrotic tissues are indicated by blue stain. Scale bars = 50 μm. (*E*) Electron microscopic images of glomeruli showed podocyte foot process fusion (indicated by arrow) in FHH compared to FHH.BN14a animals at 18 wk of age. Scale bars = 500 μm. (CL) Capillary lumen, (*) $P < 0.05$ vs. FHH, (#) $P < 0.05$ vs. BN.

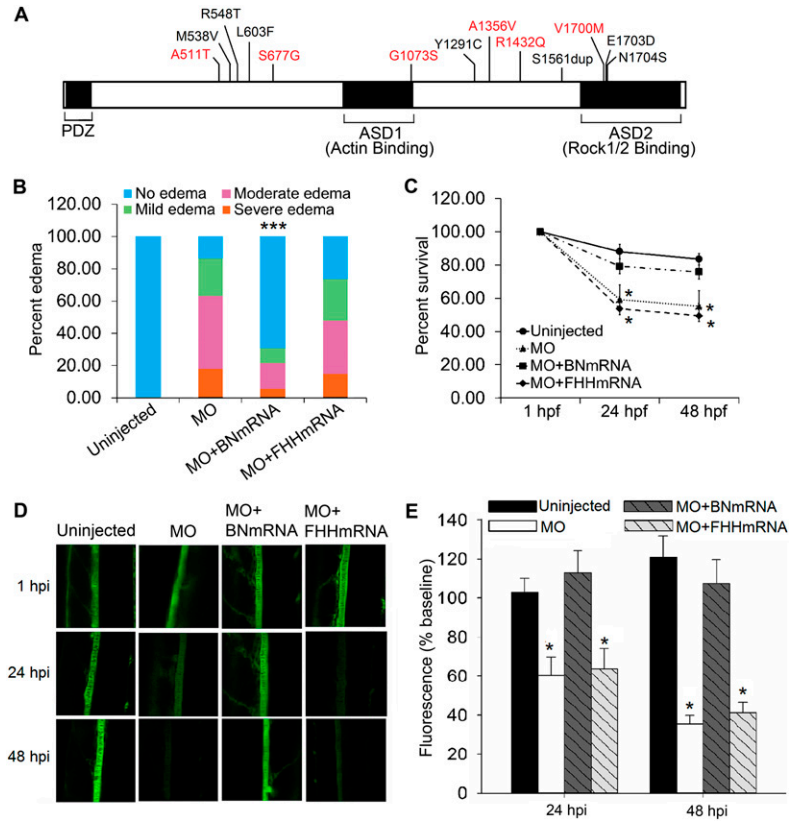

Figure 2. FHH *Shroom3* is defective and contributes to glomerular dysfunction. (*A*) Schematic representation of the rat *Shroom3* protein is shown. Vertical lines represent the amino acid variants found in FHH *Shroom3*. Variants predicted to be damaging by PolyPhen-2 are shown in red. (*B*) Co-injection of *shroom3* + *tp53* morpholino (MO) with full-length BN, but not FHH, *Shroom3* mRNA rescued the edema phenotype. ([***] $P < 0.001$ vs. MO and MO + FHHmRNA) and (*C*) cell death induced by *shroom3* + *tp53* MO ([*] $P < 0.05$ vs. uninjected control). (*D*) Representative fluorescence images of individual dorsal aorta at 1, 24, and 48 h following 70-kDa dextran injection are shown. (*E*) Co-injection with BN but not FHH *Shroom3* mRNA rescued the dextran leakage induced by knockdown of endogenous *shroom3* in zebrafish. (n = 35, 26, 23, and 22, respectively. [*] $P < 0.05$ vs. uninjected and MO + BNmRNA.)

protein. Sequence comparison of the entire congenic region identified nine missense variants in addition to those in *Shroom3* (Supplemental Table 2). The only other variants predicted to be damaging were located in genes (*Stbd1* and *Art3*) not expressed in the kidney, making *Shroom3* the most likely candidate responsible for the observed renal phenotypes in the congenic animals.

FHH *Shroom3* failed to restore glomerular function in zebrafish

In situ hybridization detected *shroom3* expression in the zebrafish pronephric glomerulus and pronephric tubule (Supplemental Fig. 2). We utilized morpholino (MO)-mediated disruption of *shroom3* in zebrafish to assess the impact of *shroom3* on kidney function. We injected one- to four-cell stage zebrafish embryos with a MO antisense oligonucleotide that caused skipping of *shroom3* exon 5 (Supplemental Fig. 3). A MO against *tp53* was co-injected to prevent potential nonspecific cell death (Robu et al. 2007). We observed that uninjected embryos developed normally; whereas *shroom3* + *tp53* MO-injected embryos predominantly (> 85%) displayed cardiac edema (Supplemental Fig. 4A), consistent with pronephros dysfunction (Kramer-Zucker et al. 2005). A fraction of the *shroom3* + *tp53* MO-injected embryos also exhibited gastrulation defects. We analyzed the respective groups using an adaptation of a previously described clearance assay to examine pronephric function (Supplemental Fig. 4B; Hentschel et al. 2007), including knockdown of *cd2ap*, a critical factor for renal glomerular function (Shih et al. 1999), as a positive control in the assay. At 24 and 48 h post-injection (hpi) of 70-kDa fluorescein isothiocyanate (FITC) dextran, we found that the FITC signal was unchanged in the dorsal aorta of uninjected controls and *tp53* MO-alone injected zebrafish (Supplemental Fig. 4C,D). In contrast, zebrafish injected with *cd2ap* + *tp53* MO or *shroom3* + *tp53* MO exhibited significant reduction in FITC signal at 24 and 48 hpi, suggesting that the glomerular size-selective property was compromised after *cd2ap* or *shroom3* knockdown, resulting in an increased rate of clearance of the 70-kDa FITC-dextran (Supplemental Fig. 4C,D).

We next investigated whether co-injection of the zebrafish *shroom3* + *tp53* MO along with *Shroom3* mRNA from either the BN or FHH rats could rescue these defects in zebrafish. Co-injection with BN, but not FHH, *Shroom3* mRNA rescued the pericardial edema and gastrulation defects associated with *shroom3* + *tp53* MO injection (Fig. 2B,C). The glomerular defects induced by knockdown of endogenous *shroom3* were also reversed by BN *Shroom3* mRNA but not the FHH *Shroom3* allele (Fig. 2D,E). These results indicate that the wild-type BN *Shroom3* allele is functional; whereas the FHH *Shroom3* allele is defective and likely contributes to CKD risk in the FHH rat.

G1073S variant disrupted the actin-binding function of the FHH *Shroom3* allele

A total of 13 protein-coding variants were identified in the FHH *Shroom3* gene, four of which fell in conserved functional domains. We constructed three BN-FHH recombinant *Shroom3* cDNAs, where the BN-specific *Shroom3* sequence was replaced by FHH alleles from nucleotides 641–3044 bp, 3044–4117 bp, or 4117–5966 bp (Fig. 3A). Co-injection of the *shroom3* + *tp53* MO with Shroom3Δ641–3044 or Shroom3Δ4117–5966 mRNA successfully reverted the dextran leakage phenotype observed upon administration of the MO alone; whereas Shroom3Δ3044–4117 mRNA did not, suggesting that at least one of the three variants (G1073S, Y1291C, and A1356V) within this region is damaging (Fig. 3B). We then constructed single-variant substitution alleles (i.e., ΔG1073S, ΔY1291C, and ΔA1356V) and found that the ΔG1073S mRNA failed to rescue glomerular leakage in the zebrafish assay (Fig. 3C,D). The G1073S variant lies in the ASD1 (Apx/Shrm Domain 1) that mediates SHROOM3 interaction with actin (Hildebrand and Soriano 1999), raising the possibility that this aspect of protein function is affected.

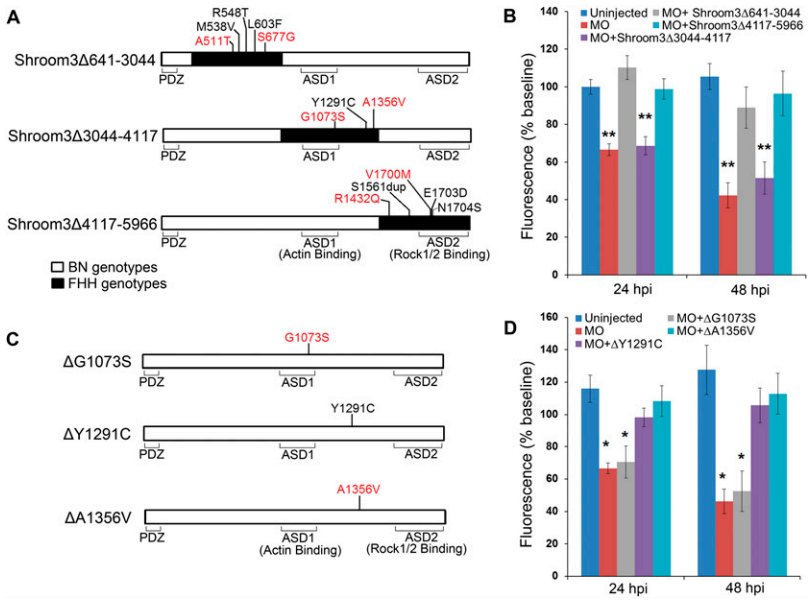

Figure 3. The G1073S variant disrupts the function of the FHH *Shroom3* gene. (*A*) Schematic of the different recombinant *Shroom3* cDNAs, where a specific region of the BN *Shroom3* sequence was replaced by FHH. (*B*) Co-injection of *shroom3* + *tp53* MO with Shroom3Δ641–3044 or Shroom3Δ4117–5966 mRNA, but not Shroom3Δ3044–4117 mRNA, rescued dextran leakage induced by the MO (n = 23, 9, 18, 28, and 15, respectively). (*C*) Schematic of *Shroom3* single-amino acid mutants created by site-directed mutagenesis. (*D*) Co-injection of ΔY1291C or ΔA1356V restored normal glomerular permeability, while ΔG1073S failed to exhibit functional rescue (n = 17, 12, 17, 23, and 21, respectively). (*) $P < 0.05$, (**) $P < 0.001$ vs. uninjected.

Coimmunoprecipitation (co-IP) of a Flag-tagged *Shroom3* allele in HEK293 cells demonstrated that the FHH *Shroom3* allele exhibited significantly lower affinity for actin than the BN *Shroom3* allele, and this defect was recapitulated by the ΔG1073S mutant allele (Fig. 4A–C). The *Shroom3* ASD2 domain mediates direct interaction with ROCK proteins (Nishimura and Takeichi 2008), which we examined using the co-IP assay. As expected, mutations in the ASD1 actin-binding domain had no effect on SHROOM3-ROCK1 interaction (Fig. 4A–C). These data demonstrate that the G1073S variant in Shroom3 may disrupt the normal glomerular filtration barrier by compromising the interaction of SHROOM3 with actin.

PI244L variant disrupted the function of human *SHROOM3* gene

GWAS have detected common noncoding variants in *SHROOM3* that are significantly associated with CKD, but the overall contribution of these variants to CKD risk is modest (Boger and Heid 2011). To identify other potential contributors to the disease risk, we tested variants in the human *SHROOM3* exon5, which harbors the ASD1 actin-binding domain, for association with nondiabetic end-stage kidney disease (ESKD) in 2465 African Americans. The clinical characteristics of the study participants and identification of sequence variants were described in Supplemental Table 3 and Methods. Based on criteria previously described (Bonomo et al. 2014), we selected 20 coding variants and three splice-site variants in the *SHROOM3* exon 5 for genotyping.

Among the coding variants, rs181194611 showed association with ESKD (P = 0.014, OR = 7.95, MAF = 0.0009) (Supplemental Table 4). This rare variant causes an amino acid substitution from a highly conserved proline to leucine at position 1244 (p.P1244L) (Fig. 5A). P1244L lies downstream from the *SHROOM3* ASD1 domain and maps to a homologous region in the mouse ortholog that is known to mediate actin interaction (Hildebrand and Soriano 1999). This variant was predicted to be functionally damaging by PolyPhen-2 (Adzhubei et al. 2010), SIFT (Ng and Henikoff 2001), and PROVEAN (Choi et al. 2012). Co-injection of *shroom3* + *tp53* MO with the nonmutated human *SHROOM3* mRNA restored glomerular function in zebrafish; whereas ΔP1244L mRNA did not (Fig. 5B,C), demonstrating that the P1244L variant indeed impairs *SHROOM3* function and is a damaging mutation that is likely contributing to the underlying pathogenesis of glomerular dysfunction.

To examine the potential role of noncoding variants in *SHROOM3*, we tested 11 *SHROOM3* noncoding variants previously associated with diabetic kidney disease in African Americans (McDonough et al. 2011) and eight noncoding variants located in transcription factor binding sites. Two common intronic SNPs (rs17002091 and rs17002201) previously identified in diabetic kidney disease exhibited association in this study (P = 0.037 and 0.015, respectively, OR = 0.83 for both SNPs) (Supplemental Table 4). No correlation was found between the two SNPs; thus they are likely distinct signals (r^2 = 0.004, D′ = 0.286). Analysis of the rs17002091 and rs17002201 haplotypes identified potentially damaging variants (rs72868158 and rs55650799, respectively), which were predicted

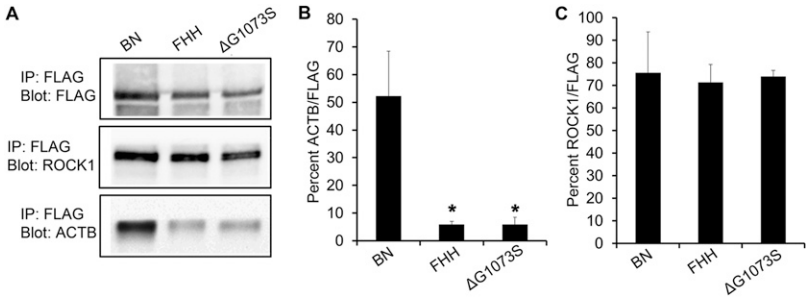

Figure 4. The G1073S variant decreases the actin-binding affinity of SHROOM3 protein. (*A*) FLAG-tagged BN, FHH, or ΔG1073S SHROOM3 proteins were overexpressed in HEK293 cells, followed by immunoprecipitation against FLAG. Immunoprecipitated lysates were immunoblotted using antibodies against FLAG, ROCK1, and ACTB. A representative Western blot of immunoprecipitated lysate is provided. (*B*) Quantification of the Western blot showed that FHH SHROOM3 and ΔG1073S mutant had significantly reduced actin-binding affinity compared to BN SHROOM3. (n = 3 per group. [*]$P < 0.05$ vs. BN.) (*C*) ROCK1-binding affinity was not different among the three alleles (n = 3 per group).

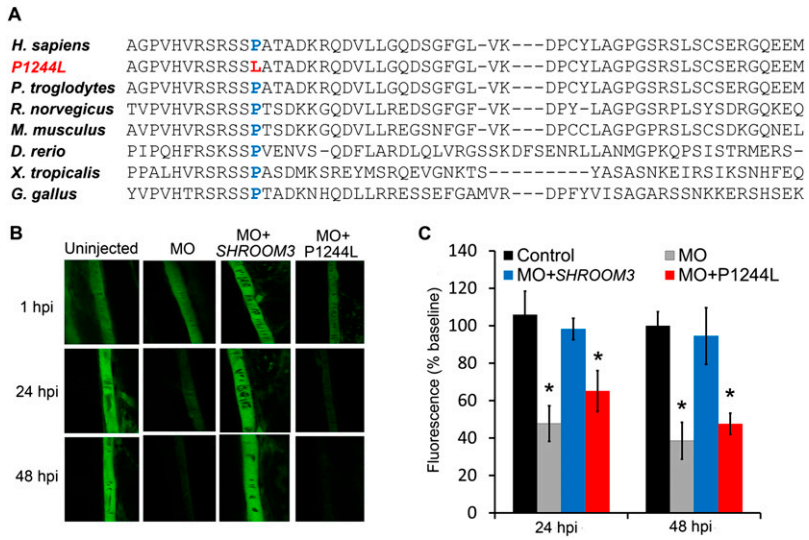

Figure 5. rs181194611 (p.P1244L) associated with nondiabetic ESKD impairs *SHROOM3* function in vivo. (*A*) Proline at the amino acid position 1244 in *SHROOM3* is evolutionarily conserved. (*B*) Representative images of dorsal aorta at 1, 24, and 48 h following 70-kDa dextran injection are shown. (*C*) Coinjection of nonmutated human *SHROOM3* mRNA restored normal glomerular permeability, while ΔP1244L failed to show functional rescue. (*) $P < 0.05$ vs. uninjected.

to disrupt transcription factor binding sites by RegulomeDB (http://www.regulomedb.org/) (Boyle et al. 2012). Testing of rs72868158 and rs72663250 (proxy of rs55650799) replicated the association previously observed, suggesting that these\ variants underlie the risk associated at this locus. Our results suggest that both common noncoding variants and rare coding variants affecting *SHROOM3* expression or function may be contributing to the risk of developing complex kidney disease in humans.

Podocyte-specific disruption of *shroom3* caused podocyte effacement in zebrafish

Injuries targeting the glomerular epithelial cells (podocytes) are a major cause of renal impairment in the FHH rat (Simons et al. 1993; Kriz et al. 1998) and proteinuric kidney diseases in humans (Faul et al. 2007; Brinkkoetter et al. 2013). Considering that *Shroom3* is expressed in the podocytes (Saleem et al. 2008; Brunskill et al. 2011), we hypothesized that it regulates glomerular permeability via its action on podocyte structure. We generated a zebrafish transgenic line carrying a *UAS:shroom3* dominant negative (*shrm3DN*) allele and crossed it with a line expressing GAL4 under the control of the podocyte-specific podocin (*nphs2*) promoter (Supplemental Fig. 5). The *shrm3DN* allele, encoding amino acids 808–1143 of the zebrafish *shroom3*, inhibited endogenous *shroom3* activity (Clark et al. 2012). This knockdown line is a useful tool for dissecting the cell type-specific role of *shroom3* and bypasses the confounding effect of global *shroom3* knockdown. We assessed the glomerular filtration barrier integrity using a dextran clearance assay shown in Figure 6A. Compared to the *podocin:Gal4* control, the *podocin:Gal4;UAS:shrm3DN* animals showed significantly decreased fluorescence at 24 and 48 hpi, indicating impaired glomerular size-selectivity (Fig. 6B,C). Electron microscopic analyses of the pronephric glomerulus revealed podocyte effacement, indicated by a reduced number of podocyte foot processes and increased foot process width (suggestive of podocyte fusion), in *podocin:Gal4;UAS:shrm3DN* animals (Fig. 6D,E). These results imply that *shroom3* is essential for maintaining the glomerular filtration barrier specifically by regulating podocyte integrity.

Discussion

Mutations targeting the podocyte actin cytoskeleton are the primary cause of many glomerular and proteinuric diseases of the kidney (Faul et al. 2007). The FHH *Shroom3* allele with a defective actin-binding domain failed to maintain glomerular integrity in the zebrafish, while the FHH *Shroom3* construct containing the functional BN-derived ASD1 domain improved overall kidney function in *shroom3*-deficient zebrafish. These data suggest that hypomorphic *SHROOM3* alleles (e.g., FHH and ΔP1244L) with altered actin-binding likely affect the mechanical characteristics of the glomerular podocyte by disrupting normal actin organization or assembly. It is worth noting that at this point, we do not know whether these alleles are truly sufficient or whether a combinatorial effect of alleles within the haplotype might also exert an effect (Flister et al. 2013).

The actin-binding function of *Shroom3* has been shown to be critical for proper morphogenesis of several epithelial tissues (Hildebrand and Soriano 1999; Haigo et al. 2003; Chung et al. 2010; Plageman et al. 2010). For example, *Shroom3* regulates apical constriction in neuroepithelial cells by recruiting existing actin fibers to the apical site of the cell (Hildebrand and Soriano 1999). Depletion of *Shroom3* in the neural plate prevents recruitment of actin, resulting in loss of the apically constricted morphology without affecting the cell polarity (Hildebrand and Soriano 1999). Thereby, it is possible that *Shroom3* regulates the function of kidney podocyte by acting as a morphogenetic regulator. Future studies will be needed to more clearly define the role of *Shroom3* in podocytes and investigate whether *Shroom3* regulates podocyte cytoskeletons through the same mechanisms observed in other epithelial cell types. *Shroom3* interacts with a number of other proteins in addition to actin (Lee et al. 2007; Nishimura and Takeichi 2008). Identification and characterization of these interactions in podocytes will be critical to gain a better understanding of how *Shroom3* affects the glomerular filtration barrier.

Using congenic rats, we showed that a locus containing the *Shroom3* gene modifies the risk of developing renal impairments. The congenic region contains 60 known and 43 predicted genes, some of which may also affect renal phenotypes. Based on sequence data, *Shroom3* appears to be the most likely candidate gene responsible for the renal phenotypes observed in the congenic animals. Transgenic rescue targeting *Shroom3* and then correcting the particular variants identified are logical future studies.

FHH rat is a well-established mammalian genetic model of CKD. This particular strain spontaneously develops proteinuria and glomerular injury associated with podocyte effacement (Kriz et al. 1998). Studies using the FHH rat have led to the discovery of genes including *Shroom3* that may also be important for kidney diseases in human (Lazar et al. 2013; Rangel-Filho et al. 2013). Sequence comparison between the FHH rat and the renal disease-resistant BN rat identified protein-coding variants in genes (*Umod,*

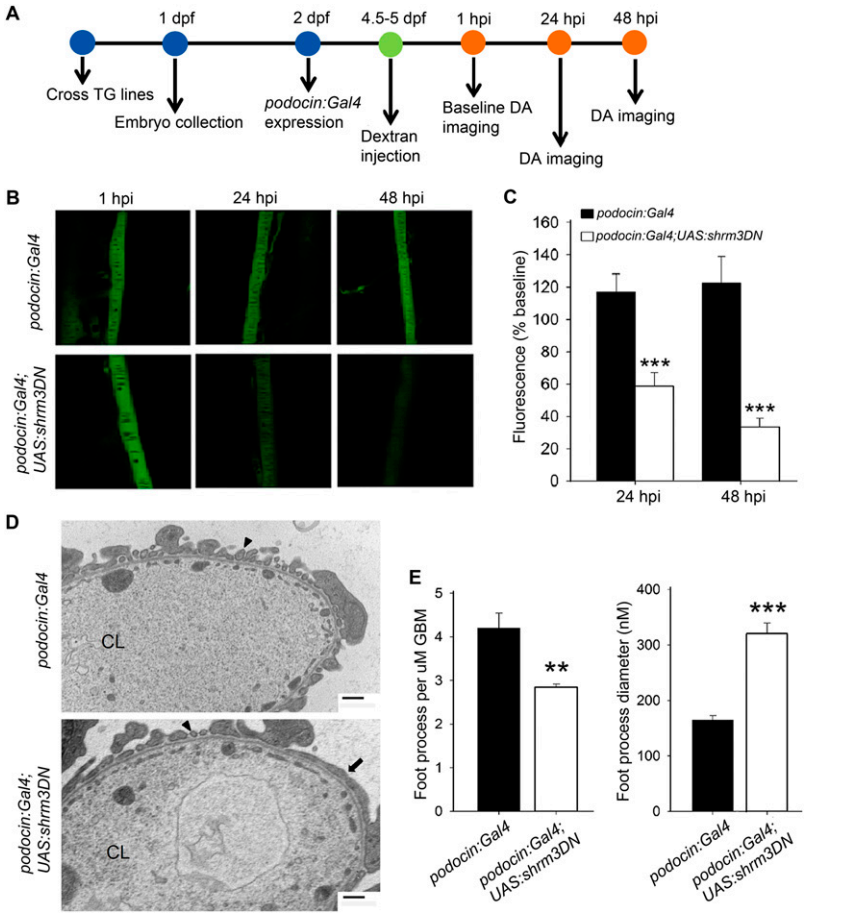

Figure 6. Podocyte-specific disruption of *shroom3* causes increased glomerular permeability and podocyte effacement in zebrafish. (*A*) Specific transgenic (TG) lines were crossed to obtain embryos expressing *podocin:Gal4* and *podocin:Gal4;UAS:shrm3DN*. The control and mutants were injected with 70-kDa FITC-labeled dextran at 4.5–5 d post-fertilization (dpf) and analyzed for dextran clearance at 24 and 48 h post-injection (hpi). (DA) Dorsal aorta. (*B*) Representative fluorescence images of individual dorsal aorta at 1, 24, and 48 hpi are shown. (*C*) *podocin:Gal4;UAS:shrm3DN* mutants had significantly decreased FITC signal at 24 and 48 hpi compared to control, indicating that the glomerular filtration barrier was disrupted. ($n = 20$ and 25, respectively. [***] $P < 0.001$.) (*D*) Electron micrograph revealed intact podocyte foot processes (indicated by arrowhead) in *podocin:Gal4* animals. Foot process effacement (indicated by arrow) was observed in the *podocin:Gal4;UAS:shrm3DN* mutants. Scale bars = 500 μm. (CL) Capillary lumen. (*E*) *podocin:Gal4;UAS:shrm3DN* had a significantly reduced number of foot processes contacting glomerular basement membrane (GBM) and increased foot process diameter compared to the control. ($n = 3$ per group. [**] $P = 0.01$, [***] $P = 0.0008$.)

Alms1, Tfdp2, Dab2, Prkag2, Ino80, Casp9, Mecom, Gnas, and *Aldh2*), in addition to *Shroom3,* which have been identified by human GWAS for CKD (Supplemental Table 5), making these interesting candidates for future studies.

Human GWAS have identified several intronic variants in the *SHROOM3* gene significantly associated with renal functional traits (Supplemental Table 1); yet, the biological role of the gene/variants has been unclear. The presented studies provide the first functional support for *SHROOM3* contributing to renal diseases in humans rather than just being a genetic marker that is correlated with the disease. Follow-up functional investigations in humans, such as fine mapping of *SHROOM3*, analysis of the gene expression or function associated with the risk haplotype, and case-control studies to test *SHROOM3* and the associated LD region, are necessary. Several lines of evidence have suggested that rare variants can play a role in human complex diseases and could account for a significant portion of the disease heritability (Pritchard 2001; Ji et al. 2008; Zuk et al. 2014). Our association analyses identified a rare variant (P1244L) in *SHROOM3* that impaired the protein function, suggesting that some rare variants in *SHROOM3* may be contributing to renal disease.

In summary, our study shows that *Shroom3* is essential for the maintenance of the glomerular filtration barrier specifically by regulating podocyte integrity. We demonstrate that *Shroom3*-mediated actin interaction is crucial for maintaining normal glomerular function, and mutations within the *Shroom3* actin-associating region may modify the risk for renal impairments. Using a combination of strategies across multiple species and data sets, we demonstrate that a rare variant of *SHROOM3* is unable to rescue renal function in zebrafish, suggesting that it could play a role in the pathogenesis of glomerular impairment in human disease. We also demonstrate that both common noncoding variants and rare coding variants affecting *SHROOM3* expression or function may be contributing to the risk of developing complex kidney disease in humans.

Methods

In situ hybridization

Zebrafish *shroom3* cDNA was PCR-amplified using a designed primer pair (Supplemental Table 6) and cloned into the pGEM-T Easy vector (Promega). The plasmid templates were linearized using SacII flanking the 5′-end of *shroom3* sequence. Linearized plasmids were subsequently used for in vitro transcription by SP6 RNA polymerase and DIG RNA labeling mix (Roche) to generate the *shroom3* antisense probe. Whole-mount in situ hybridization was performed as previously described (Thisse and Thisse 2008) on 48-hpf wild-type zebrafish embryos.

Morpholino and mRNA injections

Morpholino (MO) antisense oligonucleotides were purchased from Gene Tools. The *shroom3* MO was designed to target the intron splice-acceptor site upstream of *shroom3* exon 5. The effect of the *cd2ap* MO was previously reported (Hentschel et al. 2007). A *tp53* MO was co-injected to inhibit potential nonspecific cell death caused upon injection of the experimental MO (Robu et al. 2007). One hundred micromoles of each MO, diluted in Danieu's buffer and 0.1% phenol red, were injected in 9.2 nl per one- to four-cell stage zebrafish embryo. To assess the MO-induced splicing defects, total RNA was extracted from MO-injected or uninjected embryos with TRIzol reagent (Invitrogen), and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis kit (Fermentas). PCR was performed over the splice site to detect alternatively spliced products. The alternatively spliced PCR product was gel-extracted and sequenced. To synthesize the mRNA for the rescue experi-

ments, PCR-amplified full-length BN or FHH *Shroom3* cDNA was cloned into the pGEM-T Easy vector (Promega). Plasmids were linearized by NcoI and in vitro transcribed into mRNA using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's instructions. Four hundred picograms of either BN or FHH *Shroom3* mRNA were co-injected along with the MO into one- to four-cell stage embryos. See Supplemental Table 6 for the sequence of MO and PCR primers.

Zebrafish transgenic lines

The following lines were crossed to make the podocyte-specific *shroom3* dominant negative (shrm3DN) mutant line: Tg(*podocin:Gal4VP16*), and Tg(*dnshroom3:UAS:mCherry,myl7:eGFP*)mw47. To generate the Tg(*podocin:Gal4VP16*) transgenic zebrafish, a 5197-nt fragment of the zebrafish podocin promoter was originally cloned from BAC zC116P9 using forward 5′-TGCTACACCATT AAGGTGACCTGTG-3′ and reverse 5′-TCTGTTGTGAAGTGTCC TCT GGTG-3′ PCR primers. A 3.5-kb subfragment was generated using forward 5′-CGGTCACCGGAAGTTTATAAGTATAT GGG-3′ and reverse 5′-TCTGTTGTGAAGTGTCC TCTGGTGTTT GG-3′ PCR primers and cloned into pENTER 5′-TOPO vector (Invitrogen) and confirmed by sequencing. The final expression construct, pDestTOl2CG2;podocin:Gal4VP16:polyA, was made using the multisite gateway cloning method (Kwan et al. 2007), and transgenics were generated by plasmid co-injection with Tol2 mRNA into one-cell stage zebrafish embryos. Entry clone plasmids for *dnshroom3:UAS:mCherry; myl7:eGFP* were made using Gateway technology (Invitrogen) and Tol2-kit reagents (Kwan et al. 2007). Transgenesis of the Tol2 constructs was achieved by co-injection of transposase mRNA into one-cell stage embryos. Details of the dnshroom3 mode of action and generation of the entry plasmids have been described (Haigo et al. 2003; Lee et al. 2007; Clark et al. 2012). The following primers were used to amplify the dnshroom3 fragment: forward 5′-ATGCGATGTGTAAGTCCTGA-3′ and reverse 5′-TCAACTGTATATAAGCACTT-3′.

Zebrafish dextran clearance assay

The dextran clearance assay was adapted and modified from a previous study (Hentschel et al. 2007). For the morpholino and mRNA rescue study, 55-h post-fertilization zebrafish were used for injection of the 70-kDa FITC-labeled dextran. Zebrafish 4.5- to 5-d post-fertilization were used for injection of the dextran in the podocyte-specific *shroom3* knockdown study. Experimental zebrafish were anesthetized with tricane and embedded in 1% agarose prior to dextran injections. A total of 9.2 nl of 70-kDa dextran (1 mg/mL) was injected into the zebrafish cardinal vein. Following injection, the dorsal aorta was imaged by confocal microscopy at 1, 24, and 48 h post-injection (hpi). FITC fluorescent intensities were quantified using ImageJ software (National Institutes of Health). The 70-kDa dextran shows a low rate of filtration by an intact glomerulus and therefore should largely remain in circulation. Diminished FITC signal in the circulation at 24 or 48 hpi indicates glomerular leakage.

Transmission electron microscopy (TEM)

Zebrafish were prepared for TEM imaging as previously described (Soules and Link 2005). Briefly, the blocks were trimmed on a Leica RM2255 microtome, and ultrathin sections were cut and collected on coated grids and stained with uranyl acetate and lead citrate. Montaged images of entire cross-sectioned podocytes were captured on a Hitachi H600 at 8000×. Morphometrics of podocytes, number and size of foot processes, were scored in a masked manner where the individual scoring was unaware of the sample genotype.

Rat strain sequencing

FHH/Eur/Mcwi and BN/NHsdMcwi genomic DNA was sequenced using an Illumina HiSeq 2000 and analyzed by CASAVA version 1.8.1 (Illumina). All genomic sequences have been deposited in the NCBI BioProject under accession number PRJEB1333 (Atanur et al. 2013). Variants were analyzed by Variant Effect Predictor (http://www.ensembl.org/info/docs/tools /vep/index.html) (McLaren et al. 2010) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al. 2010).

Generation of FHH.BNI4a congenic rat and phenotyping for renal function

The FHH-14^{BN}/Mcwi consomic strain was initially derived from the FHH/Eur/Mcwi and BN/NHsdMcwi inbred strains using marker-assisted selective breeding (Mattson et al. 2007). The FHH.BN14a congenic rat strain (official name: FHH.BN-[*D14Rat98-D14Hmgc4*]/Hmgc; RGD ID: 8553187) was generated by backcrossing FHH-14^{BN}/Mcwi consomic males to FHH/Eur/Mcwi females. Thirteen- to 14-wk-old male rats were phenotyped for 24-h urinary albumin excretion and in vitro glomerular permeability using methods described previously (O'Meara et al. 2012). After urine collection for the albuminuria study, the left kidney of the experimental animals was collected and immediately fixed with 10% buffered formalin for histological analysis. Fixed tissues were stained and analyzed for glomerular sclerotic injury as previously described (O'Meara et al. 2012). For electron microscopic analysis, kidney cortex from 18-wk-old male rats was collected, prepared, and analyzed as previously described (Rangel-Filho et al. 2013). All animals were housed at the Biomedical Resource Center of the Medical College of Wisconsin (MCW) and maintained on Laboratory Rodent Diet 5001 (PMI Nutrition International Inc.) throughout the studies. Experimental protocols were approved by the MCW Institutional Animal Care and Use Committee.

Cloning of human and rat *Shroom3* plasmids

The full-length BN or FHH rat *Shroom3* cDNA was amplified using PCR and cloned into a pGEM-T Easy vector (Promega) using the manufacturer's protocol. BN-FHH recombinant *Shroom3* plasmids were generated by cloning with restriction enzymes. The pGEM-T Easy vector containing the full-length BN or FHH *Shroom3* cDNA was digested with two enzymes, AgeI and AgrAI, AgrAI and BstEII, or BstEII and NcoI (New England Biolabs). Desired plasmid fragments were gel-extracted and subsequently ligated using T4 ligase (New England Biolabs) to create Shroom3Δ641–3044, Shroom3Δ3044–4117, and Shroom3Δ4117–5966 plasmids (number indicates the base pairs of cDNA replaced by FHH alleles). To create the single amino acid substitution mutant allele, the full-length BN *Shroom3* cloned into the pGEM-T Easy vector was PCR-amplified using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) and phosphorylated primers harboring the mutated sequence. PCR products were subsequently isolated and ligated using T4 ligase. To test the human alleles, we used the commercially available myc-ddk-tagged human *SHROOM3* cDNA ORF (Origene), and the mutant allele was generated using site-directed mutagenesis. See Supplemental Table 6 for the sequence of PCR primers used in this study. All plasmids were sequenced and verified.

Protein coimmunoprecipitation (Co-IP) assay

Full-length BN *Shroom3*, full-length FHH *Shroom3*, or ΔG1073S mutant cDNA were cloned into the p3xFLAG-CMV-7.1 expression vector (Sigma) and transfected into human embryonic kidney

(HEK) 293 cells using a calcium phosphate transfection method. HEK293 cells were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% L-glutamine, penicillin streptomycin (1×), and sodium pyruvate (1×). At 24 h post-transfection, cells were collected and lysed in lysis buffer (200 mM NaCl, 20 mM Tris, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS) to extract protein. Seven hundred and fifty micrograms of protein was used for immunoprecipitation (IP) against FLAG. IP protein products were separated on 4%–20% Bio-Rad mini-PROTEAN TGX gels and transferred onto PVDF membranes, followed by overnight incubation with primary antibodies against FLAG, ACTB, and ROCK1. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies and developed with enhanced chemiluminescence reagent (Pierce). Protein bands were visualized using a ChemiDoc XRS+ Imaging System (Bio-Rad). Band intensities were quantified with ImageJ software (National Institutes of Health).

Antibodies

Primary antibodies used in this study were: mouse anti-Flag (Sigma, F3165), mouse anti-beta-actin (Abcam, ab6276), and mouse anti-Rock1 (Santa Cruz Biotech, sc-17794). Secondary antibodies used were Peroxidase-AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, 715-035-150).

Human variant association study

Coding variants in *SHROOM3* exon 5 were identified from 1000 Genomes Project and exome sequencing of an admixed African American population (Bonomo et al. 2014). Coding variants in and near the *SHROOM3* ASD1 domain were selected for genotyping using criteria previously published (Bonomo et al. 2014). Common noncoding variants in *SHROOM3* that showed prior evidence of association (McDonough et al. 2011) and promoter variants predicted to disrupt transcription factor binding by RegulomeDB (http://regulome.stanford.edu/) were also selected for genotyping. Genotyping was performed using the Sequenom MassARRAY platform (Sequenom). Genotype data were analyzed using single SNP association testing as previously described. A *P*-value of 0.05 was used as the threshold to define significance given the a priori of association between *SHROOM3* and measures of renal dysfunction. Details on sample collection, genotyping, and data analyses were previously published (Bonomo et al. 2014). This study was approved by the Institutional Review Board at Wake Forest School of Medicine.

Statistical analysis

Statistical analyses were performed using Sigma Plot 12.0 software. Data are presented as mean ± SEM. All data were analyzed by either one-way ANOVA followed by the Holm-Sidak multiple comparison test (for multiple groups) or unpaired Student's *t*-test (for two groups). Dextran clearance results obtained from the morpholino-induced knockdown and mRNA rescue failed the normality test; therefore, the data were analyzed by the nonparametric Kruskal-Wallis test followed by the Dunn's multiple comparison. The dextran clearance data obtained from podocyte-specific *Shroom3* mutant at 48 hpi failed the normality test; therefore, data were log-transformed before *t*-test analysis. Zebrafish edema incidence was analyzed by the χ^2 test.

Data access

The rat sequence variants can be downloaded from the Rat Genome Database in the Genome Browser (http://rgd.mcw.edu/fgb2/gbrowse/rgd_904/) and the Variant Visualizer (http://rgd.mcw.edu/ rgdweb/front/select.html).

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Shroom3 contributes to the maintenance of the glomerular filtration barrier integrity

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Guidelines for genetic studies in single patients: lessons from primary immunodeficiencies

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Can genetic and clinical findings made in a single patient be considered sufficient to establish a causal relationship between genotype and phenotype? We report that up to 49 of the 232 monogenic etiologies (21%) of human primary immunodeficiencies (PIDs) were initially reported in single patients. The ability to incriminate single-gene inborn errors in immunodeficient patients results from the relative ease in validating the disease-causing role of the genotype by in-depth mechanistic studies demonstrating the structural and functional consequences of the mutations using blood samples. The candidate genotype can be causally connected to a clinical phenotype using cellular (leukocytes) or molecular (plasma) substrates. The recent advent of next generation sequencing (NGS), with whole exome and whole genome sequencing, induced pluripotent stem cell (iPSC) technology, and gene editing technologies—including in particular the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology—offer new and exciting possibilities for the genetic exploration of single patients not only in hematology and immunology but also in other fields. We propose three criteria for deciding if the clinical and experimental data suffice to establish a causal relationship based on only one case. The patient's candidate genotype must not occur in individuals without the clinical phenotype. Experimental studies must indicate that the genetic variant impairs, destroys, or alters the expression or function of the gene product (or two genetic variants for compound heterozygosity). The causal relationship between the candidate genotype and the clinical phenotype must be confirmed via a relevant cellular phenotype, or by default via a relevant animal phenotype. When supported by satisfaction of rigorous criteria, the report of single patient–based discovery of Mendelian disorders should be encouraged, as it can provide the first step in the understanding of a group of human diseases, thereby revealing crucial pathways underlying physiological and pathological processes.

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In the history of clinical genetics, the delineation of novel Mendelian phenotypes often started with the description of single cases, which prompted recognition of additional patients with the same condition, defining a clinical entity and suggesting a mode of inheritance (Speicher et al., 2010). In the last few decades, genome-wide linkage analysis and candidate gene approaches have enabled the molecular genetic dissection of over 4,000 single-gene inborn errors (http://www.omim.org/statistics/entry; Antonarakis and Beckmann, 2006). Most of these advances were based on the genetic study of multiplex families, or groups of unrelated sporadic cases, or both, and progress was often accelerated by the investigation of consanguineous families. Yet, for many patients with a well-defined clinical phenotype, no disease-causing mutations can be found in any of the known disease-associated genes.

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Further, at least 1,500 Mendelian conditions lack a defined genetic etiology. Purely sporadic conditions in non-consanguineous families may also be caused by familial single-gene defects (of incomplete penetrance) or by de novo mutations (of complete penetrance) causing disease by various mechanisms (dominant-negative effect, haploinsufficiency, gain of function). Finally, many patients have a distinctive, very unusual, and possibly Mendelian phenotype that has not been described in other patients. In some cases, the discovery of the causal gene in a single patient (defined as a single patient from a single kindred) can pave the way for its confirmation in other patients. Indeed, if a first patient is not reported because it is only a single patient, a second patient may also not be reported because it would be once again the "first" patient.

Single-patient reports are common in the fields of human genetics with a tradition of mechanistic and experimental studies rooted in the availability of blood samples, such as hematology and immunology, or inborn errors of metabolism. Some investigators in other areas of medical genetics, in statistical genetics, and in experimental biology, however, question the value of genetic studies in a single patient. This point of view is illustrated in recently published guidelines (MacArthur et al., 2014) that emphasize "the critical primacy of robust statistical genetic support for the implication of new genes." Herein, we would like to emphasize the critical primacy of robust experimental support for the implication of new genes. In fulfillment of this statistical standard, the guidelines require that multiple confirmations of causality be obtained in multiple unrelated patients. Thus, multiple cases are required for the satisfaction of two of the three guidelines in "Assessment of evidence for candidate disease genes" and, in several of the other guidelines, statistical support from multiple cases is strongly encouraged (MacArthur et al., 2014). At first consideration, the logic of these recommendations is appropriate. Obviously, no reasonable person should object to the accumulation of more data to support an experimental finding. And we do not. We do agree that stringent criteria are required to avoid what statisticians call a type 1 error, the acceptance of a false hypothesis—in the current situation, the false attribution of causality based on single patients. However, insistence on too stringent accumulation of data may result in committing a type 2 error resulting in the rejection of a valid hypothesis.

We recognize that studies of single patients have limitations. First, when compared with studies of multiplex or multiple families, they do not benefit from the power of genetic homogeneity; in other words, several affected patients with the same clinical phenotype certainly provide added confidence that the altered gene is responsible for the phenotype. Second, a single affected patient does not permit one to draw firm conclusions if the candidate genotype does not display full clinical penetrance, even in the presence of a fully penetrant and relevant intermediate phenotype; in that case, the age and past medical history of the patient, as well as modifying genetic factors, may contribute to the phenotype. However, single-patient studies can be conclusive, provided there is rigorous selection of variations in silico followed by in-depth experimental validation in vitro via the dual characterization of the mutant alleles and a cellular or animal phenotype, which establishes a causal bridge between a candidate genotype and a clinical phenotype. With the notable exceptions of hematological and immunological patients (Orkin and Nathan, 2009; Ochs et al., 2014), and to a lesser extent patients with inborn errors of metabolism (Scriver et al., 2001), the description of novel gene defects in single individuals has rarely been reported. In the former patients, a blood sample is fortunately often sufficient to conduct in-depth mechanistic studies and discover relevant cellular phenotypes in erythrocytes, platelets, and any of the numerous leukocyte subsets.

In the recently published guidelines of MacArthur et al., (2014), the requirement of statistical support with an accumulation of cases is lacking in the 49/232 (21%) of monogenic primary immunodeficiencies (PIDs) first reported on the basis of a single case (Table 1). What then should be the criteria for a report based on a single patient? Based on our assessment of the 49 cases, reasonable requirements include (see Text box): in all cases, (1) population studies must indicate that the candidate genotype does not occur in healthy individuals and must have a frequency less than or equal to that predicted based on the frequency of the phenotype; (2) the genetic variants must impair, destroy, or alter the function of the gene product. In addition, for disorders that affect the function of a cell present in the patient: (3A) a patient-specific relevant cellular phenotype should be caused by the mutant allele (with its correction by complementation with the normal gene product and/or its replication by knockdown, knockout, or knock-in in relevant cells). Alternatively, for disorders that affect the development of a cell lacking in the patient: (3B) presentation of an animal model that recapitulates both the cellular and whole-organism phenotypes may replace the characterization of a relevant cellular phenotype. In either case, the third step is facilitated by the previous demonstration of genetic etiologies affecting the same physiological circuit. Together, these three steps establish a causal relationship between the candidate genotype and the clinical phenotype.

Addressing the significance and limitations of gene discovery in single patients is timely, as the NGS revolution, with whole exome sequencing (WES) and whole genome sequencing (WGS), is rapidly providing candidate variations in an increasing number of genetically undefined cases (Ng et al., 2009, 2010; Goldstein et al., 2013; Koboldt et al., 2013; Kircher et al., 2014). Although these methods may facilitate the recognition of the same genetic defects in unrelated patients, the number of single patients left without candidate genes shared by other patients will also grow. The NGS-based discovery of genetic disorders in single patients appears to have great promise in various fields of medicine, beyond hematology and immunology. Indeed, not only gradual improvements in techniques that permit transfection and knockdown of genes in primary cells and cell lines but also recent path-breaking approaches, such as iPSC (Takahashi et al., 2007; Takahashi and Yamanaka, 2013) and gene editing, especially with CRISPR/Cas9 (Marraffini and Sontheimer, 2010; Wiedenheft et al., 2012; Cong et al.,

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Table 1. Discoveries of single-gene defects underlying inborn errors of immunity in single patients

Gene product[a]	Inheritance and allele[b]	Mouse[c]	References	Citations (10/2014), ISI
Combined immunodeficiencies				
CD45	AR[g], LOF	Prior	Kung et al., 2000	179
CD3-ε	AR[g], hM/LOF	Prior	Soudais et al., 1993	62
CD3-ζ	AR, LOF[h]	Prior	Rieux-Laucat et al., 2006	48
Coronin 1A	AR[g], LOF	Concomitantly	Shiow et al., 2008	80
DNA-PK	AR[f], hM	Prior	van der Burg et al., 2009	83
CD8-α	AR[f], LOF	Prior	de la Calle-Martin et al., 2001	40
Tapasin	AR, LOF	Prior	Yabe et al., 2002	22
LCK	AR, LOF	Prior	Hauck et al., 2012	10
UNC119[d]	AD[i], LOF	Unrelated[i]	Gorska and Alam, 2012	12
CARD11	AR[f], LOF	Prior	Stepensky et al., 2013[k]	18
OX40	AR[f], LOF	Prior	Byun et al., 2013	4
Syndromic combined immunodeficiencies				
WIP	AR[f], LOF	Prior	Lanzi et al., 2012	23
RNF168	AR[g], LOF	Later	Stewart et al., 2009	295
TYK2	AR[f], LOF	Prior	Minegishi et al., 2006	244
STAT5B	AR[f], LOF	Prior	Kofoed et al., 2003	236
IKAROS	AD[i], hM	Prior	Goldman et al., 2012	5
Antibody deficiencies				
λ5	AR[g], LOF	Prior	Minegishi et al., 1998	152
Ig-α	AR, LOF	Prior	Minegishi et al., 1999a	111
Ig-β	AR, hM;LOF	Prior	Dobbs et al., 2007; Ferrari et al., 2007	30/29
BLNK	AR, LOF	Concomitantly	Minegishi et al., 1999b	188
PI3K p85α	AR[f], LOF	Prior	Conley et al., 2012	33
CD81	AR[f], LOF	Prior	van Zelm et al., 2010	102
CD20	AR[f], LOF	Prior	Kuijpers et al., 2010	97
CD21	AR[g], LOF	Prior	Thiel et al., 2012	28
Kappa chain	AR[g], LOF	Prior	Stavnezer-Nordgren et al., 1985	27
PKCδ	AR[f], LOF	Prior	Kuehn et al., 2013; Salzer et al., 2013[l]	9/8
Diseases of immune dysregulation				
CD25	AR[f], LOF	Prior	Sharfe et al., 1997	178
Fas-ligand	AD[i], LOF	Prior	Wu et al., 1996	300
NRAS[e]	AD[i], GOF	No GOF	Oliveira et al., 2007	81
Phagocyte disorders				
Rac2	AD[i], LOF	Prior	Ambruso et al., 2000	223
C/EBPε	AR, LOF	Prior	Lekstrom-Himes et al., 1999	105
P40 phox	AR[g], LOF	Prior	Matute et al., 2009	125
IL12p40	AR[f], LOF	Prior	Altare et al., 1998	263
IFN-γR1	AR[f], LOF	Prior	Jouanguy et al., 1996[m]	552
IFN-γR2	AR, LOF	Prior	Dorman and Holland, 1998	286
IRF8	AR, LOF	Prior	Hambleton et al., 2011[n]	128
Defects of innate immunity				
IκBα	AD[i], GOF	No GOF	Courtois et al., 2003	148
STAT2	AR[f], LOF[o]	Prior	Hambleton et al., 2013	3
TRAF3	AD[i], LOF	Prior	Pérez de Diego et al., 2010	81
IL17RA	AR[f], LOF	Prior	Puel et al., 2011[p]	218
APOL1	AR[g], LOF	Absent	Vanhollebeke et al., 2006	63
Auto-inflammatory disorders				
IL1RN	AR[f], LOF	Prior	Reddy et al., 2009[q]	139
Complement deficiencies				
C1qB	AR, LOF	Later	McAdam et al., 1988	41
C1qC	AR, LOF	Later	Petry et al., 1995	27

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Table 1. *(Continued)*

Gene product[a]	Inheritance and allele[b]	Mouse[c]	References	Citations (10/2014), ISI
C1s	AR, LOF	Not done	Inoue et al., 1998	11
C3	AR[f], LOF	Later	Botto et al., 1990	41
C9	AR[g], LOF	Not done	Witzel-Schlömp et al., 1997	18
Factor B	AR[g], LOF	Prior	Slade et al., 2013	1
Factor H	AR[g], LOF	Later	Ault et al., 1997	98
MASP2	AR, LOF	Not done	Stengaard-Pedersen et al., 2003	119
Ficolin 3	AR, LOF	Absent	Munthe-Fog et al., 2009	63
Total: 49 of 232 (21%) proven PIDs				

The IUIS committee for PIDs has compiled 234 genetic etiologies of PIDs into eight tables, corresponding to the eight categories in this table (Al-Herz et al., 2014). There are in fact only 232 monogenic PIDs, excluding UNC119 and NRAS deficiencies. Only loss- and gain-of-function alleles were considered to define distinct disorders; no difference was made between truly loss-of-function and hypomorphic alleles, despite their definition of distinct clinical phenotypes. We restricted our bibliographic analysis to reports of genetic lesions; some PIDs were biochemically defined before the identification of mutations, including in single patients.

[a]The 51 mutated gene products are indicated for 53 unrelated patients (two conditions were simultaneously described each in two families). With 15 exceptions (Tapasin, Lck, UNC119, WIP, Ikaros, PI3K p85α, CD81, CD20, CD21, p40 phox, IRF8, STAT2, TRAF3, APOL1, and factor B), a second or more patients were subsequently identified (references available upon request; unpublished data). In some families listed herein, one or more deceased siblings were not genetically tested.

[b]Mode of inheritance and nature of the morbid alleles. AR, autosomal recessive (bi-allelic mutations); AD, autosomal dominant; LOF, loss-of-function; hM, hypomorphic; GOF, gain-of-function.

[c]The corresponding knockout mouse was made prior to, concomitantly with, or after the human deficit was described, or not at all.

[d]The UNC119 mutation is not disease-causing as it is in fact a common polymorphism (>1%) in several human populations (http://useast.ensembl.org/Homo_sapiens/Variation/Population?db=core;g=ENSG00000109103;r=17:28546707-28552668;v=rs199714731;vdb=variation;vf=54110701).

[e]The NRAS mutation is disease-causing but was later found to be somatic, not germline, consistent with the previous discovery of NRAS germline mutations in patients with Noonan syndrome (Niemela et al., 2011).

[f]Known consanguineous family and homozygous patients (18 conditions and 18 patients). [g]Compound heterozygous patients (12 conditions and 12 patients); the others are homozygous but not known to be born to consanguineous parents (15 conditions and 16 patients). PCKδ deficiency was described simultaneously in a consanguineous and in a non-consanguineous family.

[h]Somatic mutations rescued one of the two mutant alleles in a proportion of T cells.

[i]The UNC199, Fas-ligand mutations' familial segregation were not tested, whereas the Ikaros, NRAS, Rac2, IκBα, and TRAF3 mutations occurred de novo.

[j]The UC119 mutant mouse was made previously but not studied for immunological phenotypes. The human gene had been previously shown to be important for T cell activation.

[k]Another report was published shortly thereafter yet was quoted as unpublished data in this paper (Greil et al., 2013).

[l]Another report was published shortly thereafter (Belot et al., 2013).

[m]Another report was published concomitantly in a multiplex family (Newport et al., 1996).

[n]Two patients with AD IRF8 deficiency and a different immunological and clinical phenotype were reported jointly.

[o]The STAT2 allele might be severely hypomorphic or completely loss-of-function.

[p]AD IL-17F deficiency (in a multiplex family) was reported together with AR IL-17RA deficiency.

[q]Another report was published concomitantly in a multiplex family (Aksentijevich et al., 2009).

2013; Charpentier and Marraffini, 2014), offer new and almost unlimited possibilities to provide mechanistic insights into the significance of candidate mutations in the cell types that are the most relevant to the phenotype under study. What was common practice in hematology and immunology can now be widely applied.

Single-patient discoveries in the field of inborn errors of immunity

The field of PIDs illustrates the power of single-patient genetic studies. At first glance, up to 51 of 234 PIDs (22%) were first described in single patients (with 53 patients and papers because two disorders were each reported in two unrelated patients simultaneously; Table 1). In our view, however, two single-patient studies failed to convincingly establish causality between a germline genotype and a clinical phenotype (Table 1). A disease-causing mutation in NRAS that was initially reported to be germline was subsequently found to be somatic (Oliveira et al., 2007; Niemela et al., 2011). A mutation in UNC199 that was initially reported to be rare and disease-causing is in fact a common polymorphism that does not cause disease (Gorska and Alam, 2012). Most reports were published in journals that emphasize the importance of in-depth mechanistic studies; they were highly cited. There is a trend toward an increased number of such publications over the years, with two peaks in 1995–1999 and 2010–2013, the latter explained in part by the advent of WES (Fig. 1). Most of the reports of single patients described rare disorders caused by uncommon or private genetic variations, with two exceptions (MAPS2 and Ficolin 3 deficiency). In 44 of 49 conditions, the inheritance is AR, and in the remaining five it is autosomal dominant (AD). In the patients with AD disease, the mutation was proven to be de novo in the four patients whose parents could be tested (Ikaros, Rac2, IκBα, and TRAF3; Ambruso et al., 2000; Courtois et al., 2003; Pérez de Diego et al., 2010; Goldman et al., 2012). Parental consanguinity in patients with AR inheritance was high, with up to 18 of 46 kindreds (2 disorders where reported concurrently in 2 kindreds). Homozygous lesions in the absence of known consanguinity were found in 16 patients, with the remaining 12 patients being compound heterozygous. For 36 of 49 conditions, the subsequent description of other patients corroborated the initial discovery; most of the 13 exceptions were published recently (11 after 2010), suggesting that the findings may still be confirmed. In all cases, in-depth

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Criteria that must be met to attribute a clinical phenotype to a candidate genotype in a single patient.

1. **Family studies and population studies must indicate that the patient's candidate genotype is monogenic and does not occur in individuals without the clinical phenotype (complete penetrance).**

   a. The clinical phenotype must be rare and distinctive and the candidate genotype must be monogenic.

   b. Family studies must demonstrate that the candidate genotype of the patient (which includes alleles at both loci for autosomal genes or X-linked genes in females) is not shared by other family members. In other words, there must be complete clinical penetrance, with a Mendelian mode of inheritance (AR, XR, AD, or XD).

   c. Population studies, including but not restricted to the same ethnic group, must indicate that the candidate genotype does not occur in healthy individuals tested, and that the frequencies of the candidate variants and genotype are not higher than that predicted by the frequency of the clinical phenotype.

   d. If the variant leads to a premature stop codon (nonsense, frameshift, or essential splice variants), other variants giving rise to premature stop codons must not be more frequent in the general population than predicted by the frequency of the clinical phenotype.

2. **In-depth experimental and mechanistic studies must indicate that the genetic variant destroys or markedly impairs or alters the expression or function of the gene product (or two genetic variants in the case of compound heterozygosity).**

   a. A variant in a protein-coding gene can be nonsynonymous (change the amino acid sequence) or, if synonymous, have a proven impact on mRNA structure or amount (e.g., create an abnormal splicing site). A variant in an RNA gene must affect its function (if its expression is detectable).

   b. Studies should document whether the variant changes the amount or molecular weight of the gene transcript and of the encoded protein. Ideally, this should be done in control primary cells or iPSC-derived cell lines, and not only in control immortalized cell lines.

   c. Computer programs that predict whether a missense variant is damaging are helpful but not conclusive. A variation that is not conservative and that occurs in a region or at a residue of the encoded protein that is highly conserved in evolution provides support for the hypothesis that the amino acid is functionally important.

   d. The variants must be loss or gain of function for at least one biological activity. For variants that result in an amino acid substitution, insertion, or deletion, in vitro studies should document a functional change that reveals the mechanism by which the variant causes disease. For example, the protein may be unstable, it may not bind essential cofactors, or it may not localize appropriately.

3. **The causal relationship between the candidate genotype and the clinical phenotype must be established via a relevant cellular or animal phenotype.**

   a. In all cases, the candidate gene should be known or shown to be normally expressed in cell types relevant to the disease process. These may be cells affected by the disease process, cells which produce factors needed by the affected cells or progenitors of the cell lineage affected by the disease. Some genes are broadly expressed but have a narrow clinical phenotype.

   b. For disorders that affect the function of a cell (present in the patient), experimental studies in vitro must indicate that there is a cellular phenotype explained by the candidate genotype (see c). This cellular phenotype should reasonably account for the clinical phenotype because the cell type is known to be involved in the disease process and the clinical phenotype is consistent with it. For example, if the candidate gene can be connected to a known disease-causing gene via a common cellular phenotype (e.g., mutations in a second chain of a receptor), causality is thereby established between the genotype and the clinical phenotype.

   c. The patient-specific cell type can include a convenient cell line (EBV-B cell, SV40 fibroblasts) but should also ideally include a more relevant leukocyte subset or a primary or iPSC-derived nonhematopoietic cell. This cellular phenotype must be rescued by a wild-type allele or for dominant-negative mutations by knockdown, knockout, or correction of the mutant allele. Negative dominance must be established by co-transfecting the mutant and wild-type alleles into cells deficient for the gene product. These experiments have become easier with new transfection approaches, siRNA and shRNA, and CRISPR/Cas9 editing. Alternatively or additionally, knockdown or knockout of the wild-type gene, or introduction of knock-in mutations in control cells, should reproduce the cellular phenotype.

   d. For disorders that affect the development of a cell (lacking in the patient), a cellular phenotype is difficult to establish. The candidate gene can be connected to a known disease-causing gene via a common mechanism. Causality is thereby suggested between the genotype and the clinical phenotype. In this and other cases, when the candidate gene governs a novel circuit, an animal model in vivo must, however, indicate that there are causally related phenotypes that mimic the patient's phenotypes (molecular, cellular, and clinical) and are explained by the candidate genotype. A biological phenotype underlying the patient's clinical phenotype must be replicated in the mutant animal (e.g., IgA deficiency underlying a specific infection).

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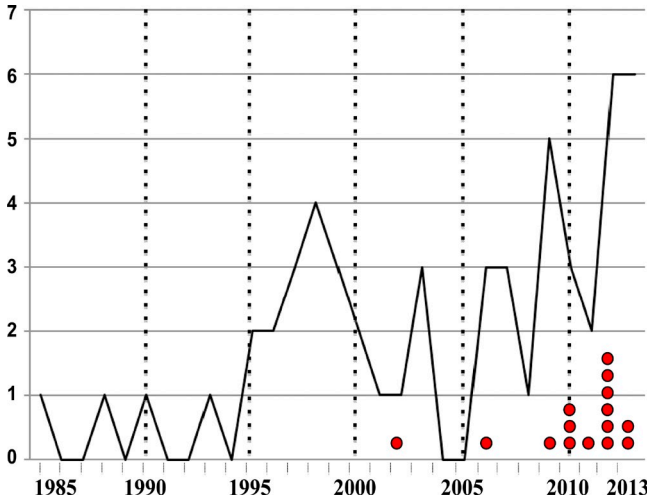

Figure 1. Distribution of single-patient inborn errors of immunity reported per year. The red dots indicate conditions that have not yet been reported in a second patient. It is notable that the number of single-gene defects reported per year is increasing with time, with two peaks, one in the years 1995–1999 and another in 2010–2013. The first peak (13 patients) corresponds mostly to the discovery of genetic etiologies of classical PIDs that had been long clinically delineated, all discovered by newly developed genetic tools for linkage analysis and a candidate gene approach. The second peak (15 patients) benefited from the advent of NGS (PI3K p85α, CARD11, OX40, and PCKδ deficiencies in a total of 5 patients) but also reflected the growth of the field and the exploration of novel phenotypes (e.g., IRF8, STAT2, and Ikaros deficiencies).

mechanistic studies in the patients' myeloid or lymphoid cells proved the deleterious nature of the mutations found. In turn, the pathological nature of most alleles was documented on immunological grounds. For some genes, a connection with a known morbid gene was established by the characterization of a cellular phenotype. For most genes, the known role of the mouse ortholog provided evidence that the mutation was responsible for the clinical and immunological phenotype observed (Table 1). In these and other instances, the gene was often connected with known human pathways, and the morbid nature of the mutation was rarely provided on the sole grounds of genetic and experimental data.

What did we learn from these 49 PIDs?

The discovery of novel monogenic defects in single patients has often offered novel biological and pathological insights. For example, there was no previous evidence in mice or humans pointing to the role of RNF168 in DNA repair and immunity (Table 1). It is its defective biological function in human mutated cells that established both its morbid nature and physiological role, with RNF168 deficiency underlying RIDDLE syndrome (Stewart et al., 2009). For four components of the complement pathway, the human defect was documented before the development of the corresponding mutant mouse, and its disease-causing nature was established thanks to biochemical studies using human plasma (McAdam et al., 1988; Botto et al., 1990; Petry et al., 1995; Ault et al., 1997). In three instances, the mouse mutant has not been generated (Witzel-Schlömp et al., 1997; Inoue et al., 1998; Stengaard-Pedersen et al., 2003). In the case of APOL1 (Vanhollebeke et al., 2006) and ficolin 3 (Munthe-Fog et al., 2009), there is no mouse ortholog (Table 1). In two instances, Coronin and BLNK deficiency, the mouse and human defects were reported jointly (Minegishi et al., 1999b; Shiow et al., 2008). The other cases were no less interesting, as the associated phenotypes were often surprising when compared with the corresponding mutant mice, sometimes in terms of the impact of the mutation on the immune response and more often in terms of its impact on clinical phenotypes, particularly susceptibility to specific infections. For example, the role of OX40 in T cells had been characterized in mice but its role in human immunity against HHV-8 was only established by the demonstration of OX40 deficiency in a child with Kaposi sarcoma (Byun et al., 2013). Monogenic predisposition to other specific infectious diseases is increasingly documented (Alcaïs et al., 2010; Casanova and Abel, 2013). Hypomorphic (DNA-PK, CD3ε, Igβ, and Ikaros; Soudais et al., 1993; Dobbs et al., 2007; van der Burg et al., 2009; Goldman et al., 2012), gain-of-function (IκBα; Courtois et al., 2003), and/or heterozygous (TRAF3, Ikaros, Fas-ligand, Rac2, and IκBα; Wu et al., 1996; Ambruso et al., 2000; Courtois et al., 2003; Pérez de Diego et al., 2010; Goldman et al., 2012) human mutations also revealed phenotypes not seen in mice bearing two null alleles. Overall, an important added value of the genetic dissection of human PIDs, in single patients or multiple patients, besides its direct medical impact, is that it enables an analysis of immunology in natural as opposed to experimental conditions (Casanova and Abel, 2004, 2007; Quintana-Murci et al., 2007; Casanova et al., 2013).

Fully penetrant autosomal and X-linked recessive traits

What general lessons can we draw from the study of single patients with PIDs? First of all, what are the Mendelian modes of inheritance (in the sense of full penetrance) that are most appropriate for single-patient studies? Autosomal recessive (AR) inheritance is appropriate, especially in consanguineous families (Lander and Botstein, 1987), as one can benefit from linkage information and focus on homozygous mutations. The larger the number of healthy siblings, the easier it is to select candidate variations. The sequencing data can be filtered quickly to identify these mutations. Information increases with the level of the consanguinity loop—i.e., the more distant the parental relationship, the more accurate the linkage mapping. The large fraction of homozygosity in the patient (e.g., expected value of 6.25% if born to first cousins) increases the background noise of homozygosity for other, non–disease-causing, genomic variants. Other caveats should be kept in mind. Consanguineous families are not protected from X-linked diseases, compound heterozygous alterations, or de novo mutations. Moreover, if the family is highly consanguineous, the patient may be homozygous for uncommon, albeit non-rare, genetic variants that modify the phenotype. Conversely, non-consanguineous families reduce the background but leave the mode of inheritance uncertain. Homozygosity in a patient born

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to parents not known to be related is suggestive of AR inheritance due to cryptic consanguinity. Compound heterozygosity is even more suggestive of AR inheritance, as it is rare and deserves special attention. In that regard, the trio design, NGS analysis of the patient and both parents supports the search for compound heterozygous mutations. Finally, X-linked recessive (XR) inheritance is possible with a focus on hemizygous mutations in males. In the latter two cases, the existence of a de novo mutation provides further evidence given the relatively small number of coding de novo mutations per genome (Sanders et al., 2012; Veltman and Brunner, 2012; Ku et al., 2013). Obviously, incomplete penetrance and the impact of modifiers clearly cannot be studied in single patients (Cooper et al., 2013).

De novo mutations underlying dominant or XR traits

The trio design enables one to detect de novo mutations, which are more likely to be disease-causing and certainly easier to incriminate in single patients than inherited variations. A de novo mutation provides strong but not conclusive evidence that the variant is related to the clinical phenotype. Highly deleterious, distinctive phenotypes that occur in outbred populations may be due to heterozygous or hemizygous de novo mutations (Boisson et al., 2013). In such instances, the clinical penetrance is typically complete. It is estimated that 50–100 new sequence variants can be found in the genome of every individual. However, most of these genome variants have no functional consequences because they do not change the amino acid sequence of coding regions or they occur in noncoding or nonregulatory regions. On average, only 1 or 2 de novo mutations can be found in each exome (Sanders et al., 2012; Veltman and Brunner, 2012; Ku et al., 2013). These new mutations may cause a phenotype that is so severe that it is rarely passed on to the next generation. Variants that are found in the patient but not the parents are excellent candidate disease-causing mutations, especially if they are in a plausible gene (see section below). The de novo mutation alone may underlie an AD or XR disorder (or as discussed above an AR disorder if coupled with an inherited mutation on the other allele). It is important to test multiple cell types, hematopoietic and non-hematopoietic, as the apparently de novo mutation detected may not be germline but somatic, in which case it may remain disease-causing, albeit not underlying a monogenic trait, as shown for NRAS (Table 1). The search for de novo mutations alone justifies the strategy of sequencing trios, which is quite powerful and fits well with single patient investigation (Veltman and Brunner, 2012).

Focus on rare phenotypes and rare genotypes

The genotype and allele frequencies are important factors to consider when selecting candidate variants (Kircher et al., 2014). It is not too difficult to select candidate disease-causing genetic lesions in a single patient because the most common reason for a study to be restricted to a single patient is precisely that the disease is extremely rare. A rare phenotype is likely to be due to heterozygosity or homozygosity for a very rare or private allele. In current practice, one can search for the variant in multiple online databases. Advances in NGS have made it possible to collect information on the frequency of genetic variations in a much larger number of individuals of various ethnicities. Public databases of variants (dbSNP, HapMap, 1000 Genomes, and NHLBI "Grand Opportunity" Exome Sequencing Project [GO-ESP, https://esp.gs.washington.edu/]; Altshuler et al., 2010; Abecasis et al., 2012; NCBI Resource Coordinators, 2014) and disease-causing variants (HGMD; Stenson et al., 2014) typically include data on the genetic variability of between 10,000 and 100,000 individuals. This is valuable but occasionally not sufficient. The patient may be from an ethnic group that is under-represented in available databases. Further, some DNA sequences were not well enough covered using older approaches to catalog variants. An in-house database that includes data from over 500 ethnically matched DNA samples analyzed using the same technology is valuable. Moreover, private databases of neutral datasets are being developed at institutions that are taking advantage of NGS to search for disease-causing gene variants. With very rare phenotypes, it is helpful to select private variations in single patients. However, except in the specific context of de novo mutations, there are still a substantial number of private (or very rare) variants in any exome data. One should consider the genotype frequency, which is more relevant than the allele frequency. A very rare AR condition may be caused by a mutant allele that is rare, but not private to the family studied, and found in control heterozygotes. The next step is to prioritize these variants by further in silico studies to select the most plausible one.

Searching for plausible genes

Information about the mutated genes is also crucial in selecting variations and filtering out others. There are at least three ways by which a mutated gene can be selected as a candidate disease-causing gene in a single patient. First, the gene may encode a protein that belongs to a pathway already implicated in patients with the same phenotype. The relationship between these genes may be distant and indirect, and tools such as the human gene connectome can be helpful in revealing their connectivity (Itan et al., 2013). In other words, there can be physiological homogeneity behind genetic heterogeneity. Second, there is increasingly detailed information in various databases regarding the expression of human genes in a wide array of human cell lines, cell types, tissues, and organs (GTEx Consortium, 2013; Rung and Brazma, 2013). Transcripts for the gene of interest are likely to be expressed in the tissue affected by the phenotype or tissues that are known or could reasonably be expected to influence the phenotype. However, mutations in broadly expressed genes occasionally result in a phenotype that is highly tissue specific (Boisson et al., 2013). Third, genes crippled with deleterious mutations in the general population are unlikely to be causative of any rare phenotype with complete penetrance, as assessed for example with the gene damage index (unpublished data). Knowing the degree of purifying selection operating on the genes carrying variations in the patient under study is also helpful (Barreiro and Quintana-Murci,

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2010; Quintana-Murci and Clark, 2013). The extent of purifying selection is now known for most human genes (Barreiro and Quintana-Murci, 2010; Quintana-Murci and Clark, 2013). Mutations in genes under tight purifying selection represent more likely culprits for rare diseases, especially for diseases that are life-threatening in childhood, and more so for AD than AR modes of inheritance (X-linked recessive being probably in between). To further incriminate haploinsufficiency for AD and XD traits that are life-threatening before reproductive age, the gene must be under purifying selection.

Searching for plausible mutations

The predicted impact of the mutation itself is also important, as some lesions are predicted to be more damaging than others (Kircher et al., 2014). As of now, protein-coding gene exonic mutations are more easily implicated than regulatory mutations or mutations in RNA-coding genes or in intergenic regions. Upcoming progress may facilitate the study of such mutations in single patients in the future. In protein-coding genes, UTR and synonymous variations are difficult to incriminate in single patients, even though they can interfere with splicing and other regulatory processes. In contrast, nonsense mutations affecting canonical splice site residues, in-frame and out-of-frame insertions and deletions, and mutations of the stop codon (stop loss) are most likely to be deleterious, although some can be hypomorphic, for example due to reinitiation of translation. The impact of missense mutations, which form more than 90% of rare and common nonsynonymous variations (and a smaller proportion of pathogenic mutations; Tennessen et al., 2012), is less easily predictable and has therefore received much attention. Some missense mutations are intrinsically more disruptive than others. Moreover, a missense mutation at a residue or in a domain that is highly conserved throughout evolution is probably damaging. Several software programs, such as Sorting Intolerant From Tolerant (SIFT; Kumar et al., 2009) and PolyPhen2 (Adzhubei et al., 2010), have been developed to predict the pathogenicity of missense mutations based on a combination of biochemical and evolutionary data (Li et al., 2013). Although mutations predicted to be loss-of-expression and/or loss-of-function are more readily convincing, amino acid substitutions may be more interesting because they can provide insights into function of a particular domain or the effects of decreased but not absent protein function, or even into the impact of a gain of function. Overall, the predicted impact of any given mutation influences its selection as a candidate lesion to be functionally investigated in single patients (Kircher et al., 2014).

Biochemically deleterious alleles support a causal relationship

In the PID field, the ability to experimentally validate the mutation in leukocytes can document its causal role based on a single patient observation. The same principle can now be applied to other fields, with equally high expectations. The report of a mutation that fulfills the in silico criteria listed above is likely to be disease-causing but must be confirmed by in-depth, functional, and mechanistic studies. The first step consists of testing the impact of the mutation on the expression of the gene product. This is easily done with protein-coding genes, even if there is no antibody to the protein of interest, using N- or C-terminal tags and a variety of easily transfectable recipient cells, which may not necessarily be relevant to the clinical phenotype. A biochemically deleterious allele, in terms of protein expression, is often the first experimental evidence that the variation is disease-causing. The subcellular trafficking, distribution, or location of the mutant proteins is often informative, although overexpression studies in irrelevant cell lines may be misleading. Expression studies can be performed regardless of the nature of the gene and even in the absence of patient's primary cells or cell lines. In contrast, functional studies by gene transfer can be more difficult to conduct, as they require at least some knowledge regarding the function of the mutated gene. This biochemical step, which does not necessitate any cells from the patient, is important for the validation of candidate variations. It is currently more difficult to study the expression of RNA genes by gene transfer; this is a poorly investigated topic due to the rarity of such pathological lesions to date (Batista and Chang, 2013), which certainly deserves more effort in the future. Biochemical studies of the candidate mutant allele should ideally be compared with other mutants, rare or common, found in individuals without the phenotype under study and serving as negative controls.

Relevant cellular or animal phenotypes support a causal relationship

Relevant cells from the patient should demonstrate a functional abnormality that is caused by the mutant allele or alleles and that can explain the clinical phenotype. For loss-of-function mutations, introduction of a wild-type copy of the gene of interest should correct the cellular phenotype, unless the mutation is dominant-negative. Similarly, validation of the disease-causing role of gain-of-function or dominant-negative mutations may be obtained by introducing a mutated copy of the gene into wild-type cells. If the mutant allele is dominant by haploinsufficiency, it can be rescued by overexpression of the wild-type and a knockdown of the wild-type allele in control cells can also be informative. However, these approaches, albeit valid, suffer from the limitation that it is hard to maintain endogenous regulation and full control of the level of expression of the transfected/transduced gene. Recently, the CRISPR/Cas9 technology has opened new perspectives, as it allows the knock-in of the appropriate mutation in one or both alleles of the gene in control cells to mimic the abnormal phenotype, or to correct the mutant alleles in the patient's cells (Marraffini and Sontheimer, 2010; Wiedenheft et al., 2012; Cong et al., 2013; Charpentier and Marraffini, 2014). This approach adds much confidence to the proposed causative relationship. These mechanistic studies have traditionally been easier with a blood sample or EBV-B cell lines, which accounts for the prominent role of hematology and immunology in the development of human molecular genetics (Speicher et al., 2010). Dermal fibroblasts have been used in various fields. More recently, the iPSC technology (Takahashi et al.,

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2007; Takahashi and Yamanaka, 2013), which enables the mechanistic study of many cell types that can be affected by disease, has opened up new perspectives in various fields. It has become possible to study patient-specific disease phenotypes in iPSC-derived neurons (Ming et al., 2011), hepatocytes (Schwartz et al., 2014), cardiomyocytes (Josowitz et al., 2011), or respiratory epithelial cells (Huang et al., 2014). This approach even enabled the study of nonhematopoietic PIDs, with the demonstration of impaired intrinsic immunity and enhanced HSV-1 growth in patient-specific, iPSC-derived TLR3-deficient neurons and oligodendrocytes (Lafaille et al., 2012). Along with biochemical studies, investigation of the specific functional consequences of a given mutation in the appropriate cell type (and correction thereof) represents the most important step in the experimental validation of candidate variations. In turn, the cellular phenotype can be causally related to the clinical phenotype in at least two ways. It can be shared by patients bearing mutations in a known disease-causing gene (e.g., mutations in another chain of the same receptor or molecular complex, or in another molecule along the same signaling pathway). Alternatively, it may be novel yet provide a plausible molecular and cellular mechanism of disease. If there is no relevant cellular phenotype, animal models can also validate the disease-causing effect of a genotype, if they recapitulate the human whole-organism phenotypes. The animal models can also serve to connect a cellular and a whole-organism phenotype. Overall, with the stringent in silico and in vitro criteria defined above, we argue that single-patient studies can be illuminating for the study of rare, Mendelian disorders in hematology, immunology, and beyond.

Concluding remarks

We have highlighted some criteria that facilitate the genetic study of single patients, particularly in families with fully penetrant AR traits and de novo mutations. In this paper, the concept was illustrated by a review of discoveries in the field of PIDs. The study of patients with unique conditions is important, both for purely clinical reasons and for increasing our understanding of physiology. Patients with a unique set of unusual findings need a genetic diagnosis. They cannot be ignored (Mnookin, 2014). Publication of these cases is also an efficient way to find and help other patients with the same phenotype and the same disease-causing gene. Discovering and reporting single-gene disorders in single patients is also important for biological reasons that go beyond the specific patient. It is not often recognized that murine knockout models are genetically more questionable than single patient studies. The murine phenotype is typically associated with and tested in a single genetic background under defined (and for this reason also unrepresentative and potentially misleading) experimental conditions (Andrews et al., 2013). The animals are therefore more likely to be homozygous for modifying genetic factors. Single patients are not 100% homozygous, making their phenotype more robust. Only 6.25% of the genome of patients born to first-cousin parents is homozygous. Interpretation of data from knockout mice is further complicated by the fact that the mice are reared in an environment that is tightly controlled. The patients' phenotype occurs in natura, as opposed to experimental conditions (Casanova and Abel, 2004). The phenotype of patients with bi-allelic null mutations therefore often differs in informative ways from mice with null mutations in the same gene. Moreover, genetic variations that are found in patients are also more diverse and thus more illuminating than mutations created experimentally in the laboratory, although ENU mutagenesis also generates hypomorphic and hypermorphic variants (Andrews et al., 2013).

As shown with PIDs, it may be weeks, months, years, or even decades to find a second patient with the same phenotype caused by a mutation in the same gene. Of course, both international collaborations and the publication of a first patient may hasten this process. What is more challenging, rare, and informative is paradoxically a patient with a different phenotype, caused by the same mutation at the disease locus. An example can be found in the realm of infectious diseases because the patient must be exposed to the organism to be at risk. Ascertainment bias, i.e., the risk of diagnosing a given genotype only in patients sharing the phenotype of the first case, is probably a more serious problem than the (obviously nonexclusive) uncertainty of causal relationship between a genotype and a clinical phenotype in single patients. In studying single patients, one should keep in mind that there are no truly Mendelian disorders because humans, like other species, are not single-gene organisms and because environment affects the phenotype. One should consider the study of single patients in a Darwinian perspective, with Mayr's population thinking, as opposed to essentialism (Mayr, 1988; Mayr, 1991) and consistent with Garrod's concept of chemical individuality of man (Garrod, 1931; Bearn, 1993). One should attempt to establish a causal relationship between a genotype and a phenotype in a unique individual, being aware that the same genotype may cause another phenotype in another patient and that the same phenotype in another patient may be caused by another genotype. The three causal relationships would all be correct. In the vast majority of cases, however, replication in other patients has been observed, and single-patient discoveries, especially in the fields of hematology and immunology, in PIDs in particular, have stood the test of time (Table 1).

Genetic heterogeneity in human populations is such that some newly discovered single-gene inborn errors may be reported in only a single patient for years or even decades. The dissection of sporadic but genetically homogenous traits is difficult, but that of sporadic heterogeneous traits is even more difficult. Needless to say, the search for lesions found in multiple patients and multiple kindreds does greatly facilitate the genetic analysis of human conditions. However, the extreme diversity of human genetic variation is not only reflected in genetic heterogeneity of well-defined and homogeneous phenotypes. In fact, each patient is unique because of the heterogeneity of genetic variants and unique environmental history. Again, the concepts of population thinking and chemical individuality are essential when reflecting on this question (Garrod, 1931; Mayr, 1988, 1991; Bearn, 1993). Because of

variability in clinical presentations, there are only patients; there are no diseases. Or, there are as many diseases as patients. Illnesses are designated by specific terms by default. NGS is pushing medicine further in that direction—we increasingly discover unique conditions and, decreasingly, universal diseases. Single patients and their families deserve this attention to unmet needs (Mnookin, 2014), and the construction of the biomedical edifice at large also deserves this effort. A single patient is a fragile bridge between two worlds, between basic scientists and practitioners. It would be unfortunate if interpretation of the guidelines proposed by MacArthur et al. (2014) to include insistence on multiple cases with similar defects results in inhibition of reports of novel monogenic inborn errors documented with only a single case. If none of the single cases are published, the only way a new entity would be reported is if at least two cases came to the attention of investigators at the same time. Consistent with a long tradition in hematology and immunology, in-depth mechanistic studies of appropriate cellular phenotypes in a single patient in relevant cell types can provide a causal bridge between a genotype and a clinical phenotype.

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Genomic Medicine IX Meeting

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Leslie G. Biesecker, MD – Dr. Biesecker is the Senior Investigator for the Genetic Disease Research Branch with NHGRI. I direct two translational research groups, a rare disease group that currently focuses on the etiology and treatment of segmental overgrowth disorders and a second group that focuses on predictive genomic medicine and secondary findings.

Carol Bult, PhD – Dr. Bult is the Professor and Cancer Center Deputy Director for the Mammalian Genetics at the Jackson Laboratory for Genomic Medicine. The primary theme of my personal research program is "bridging the digital biology divide," reflecting the critical role that informatics and computational biology play in modern biomedical research. I am a Principal Investigator in the Mouse Genome Informatics (MGI) consortium that develops knowledgebases to advance the laboratory mouse as a model system for research into the genetic and genomic basis of human biology and disease (http://www.informatics.jax.org). Recent research initiatives in my research group include computational prediction of gene function in the mouse and the use of the mouse to understand genetic pathways in normal lung development and disease. My institutional responsibilities at The Jackson Laboratory include serving as the Deputy Director of the Cancer Center and as the Scientific Director of our Patient Derived Xenograft (PDX) and Cancer Avatar program. The PDX program is a resource of deeply characterized and well-annotated "human in mouse" cancer models with a focus on bladder, lung, colon, breast and pediatric cancer. This resource is a powerful platform for research into basic cancer biology (such as tumor heterogeneity and evolution) as well as for translational research into mechanisms of therapy resistance and therapeutic strategies to overcome resistance.

Rex Chisholm, PhD - Dr. Chisholm is the Adam and Richard T. Lind Professor of Medical Genetics in the Feinberg School of Medicine and professor of Cell and Molecular Biology and Surgery. He was the founding Director of the Center for Genetic Medicine. Since 2007 he has served as Vice Dean for Scientific Affairs in the Feinberg School. In October 2012 he was also appointed Associate Vice President for Research of Northwestern University.A faculty member at Northwestern University since 1984, Chisholm is author of over 100 peer reviewed scientific publications. His research focuses on genomics and bioinformatics. Chisholm leads a major DNA biobanking effort at Northwestern University, NUgene (www.nugene.org). NUgene enrolls research participants in a study focused on investigating the genetic contributions to human disease, therapeutic outcomes and gene-environment interactions. NUgene is a participant in the NHGRI-funded eMERGE network (www.gwas.net) – a network of electronic medical record (EHR) linked biobanks. The goal of his current eMERGE network project is to establish a program for genomics-informed personalized medicine in partnership with Northwestern's health care affiliates. He is also Principal Investigator of dictyBase (dictyBase.org), the NIH-funded genome database for the cellular slime mold, Dictyostelium and is an NHGRI funded member of the gene Ontology Consortium (www.geneontology.org). Chisholm has served on numerous advisory boards and NIH study sections, including a four year term as member of the National Advisory Council for Human Genome Research. His research has been supported by the National Institutes of Health, the American Cancer Society, American Heart Association.

Gregory Cooper, PhD – Faculty Investigator for HudsonAlpha Institute for Biotechnology. Dr. Cooper calls himself "quantitatively inclined." So as a faculty investigator at the HudsonAlpha Institute for Biotechnology, Cooper is at home with genomics — the intersection of science and math. Cooper's research specializes in evolutionary and functional annotation of the genome. For example,

in one strategy he compares the human genome to other mammals to find out which sites might be conserved — or kept the same — across different species. If a region in the human genome is the same as other mammals, that similarity could be an indicator that those sites are important to our health and prevented from changing by natural selection. “Looking at genetics through an evolutionary lens is really powerful because evolution doesn’t care what the molecular activity is,” Cooper says. “Evolution only cares about what the physical trait is. And that’s what we care about.” Cooper cares about those physical traits because his research also looks at direct disease applications, especially conditions that involve intellectual disability and other developmental problems that affect children. Often, Cooper says, in children with developmental delays who are born to unaffected parents, the child’s condition is the result of a genetic change that happened during egg or sperm formation. In those cases, Cooper can subtract the parents’ genetic information from the child’s. He is left with the changes that are prime suspects for causing the child’s symptoms. Cooper came to HudsonAlpha in 2010 after receiving his PhD in genetics from Stanford University and finishing postdoctoral work at the University of Washington in Seattle. He never wanted to be a clinician, but he is excited to see his research applied to human health. “It’s thrilling,” he says, “to be able to say that basic research has now led to the ability to take a genome, extract something useful out of it and find a genetic variant that causes somebody to be sick.”

Nancy Cox, PhD – Director at Vanderbilt Genetics Institute and Division of Genetic Medicine. Nancy Cox is a quantitative human geneticist with a long-standing research program in identifying and characterizing the genetic component to common human diseases; current research is focused on large-scale integration of genomic with other “-omics” data as well as biobank and electronic medical records data.

Douglas Fowler, PhD – Dr. Fowler is a Principal Investigator, Bioinformatics, Biotechnology, Biochemistry at University of Washington. The Fowler lab works to develop experimental and computational methods to phenotype mutations, particularly in the context of cancer. Our approach is to measure the functional consequences of all possible single mutations in a protein of interest in a parallelized fashion. We then use the resulting data to make a comprehensive sequence-function map, and to train generalized variant effect predictors.

Eric D. Green, M.D., Ph.D - is the Director of the National Human Genome Research Institute (NHGRI) at the National Institutes of Health (NIH), a position he has held since late 2009. Previously, he served as the NHGRI Scientific Director (2002-2009), Chief of the NHGRI Genome Technology Branch (1996-2009), and Director of the NIH Intramural Sequencing Center (1997-2009). Dr. Green received his B.S. degree in Bacteriology from the University of Wisconsin-Madison in 1981, and his M.D. and Ph.D. degrees from Washington University in 1987. During residency training in clinical pathology (laboratory medicine), he worked in the laboratory of Dr. Maynard Olson from 1988-1992, launching his career in genomics. From 1992-1994, he was faculty member at Washington University School of Medicine. In 1994, he joined the newly established NHGRI Intramural Research Program. While directing an independent research program for almost two decades, Dr. Green was at the forefront of efforts to map, sequence, and understand eukaryotic genomes; his work included significant, start-to-finish involvement in the Human Genome Project. As Director of NHGRI, Dr. Green is responsible for providing overall leadership of the Institute’s research portfolio and other initiatives. He has led NHGRI in broadening its research mission by designing and launching a number of major programs to accelerate the application of genomics to medical care.

Melissa Haendel, PhD – Dr. Haendel is the Director of the Ontology Development Group and the Associate Professor at OHSU Library and the Department of Medical Informatics and Clinical Epidemiology. Principal Investigator of the Monarch Initiative.

Gail Herman, MD, PhD – Dr. Herman is a Professor in the Molecular and Human Genetics Department at the Research Institute at Nationwide Children’s. Dr. Herman is a Professor in the Center for Molecular and Human Genetics, The Research Institute at Nationwide Children’s Hospital, and the Dept of Pediatrics, The Ohio State University College of Medicine. She received her medical degree and a PhD in biochemistry from Duke

University and completed a residency in pediatrics and a fellowship in genetics at Baylor College of Medicine. She is board-certified in pediatrics and clinical and biochemical genetics. Her research focuses on the genetics of selected developmental disorders, including autism and mouse models for cholesterol biosynthesis disorders. She has been the Principal Investigator on a Department of Defense funded multisite translational study to develop the Central Ohio Registry for Autism (CORA) and perform molecular studies to identify additional autism candidate genes. Dr. Herman is also the Past-President of the American College of Medical Genetics and Genomics.

Howard Jacob, PhD – Executive Vice President for Genomic Medicine; Chief Medical Genomics Officer; Faculty Investigator at HudsonAlpha Institute for Biotechnology. As a faculty investigator and the executive vice president for genomic medicine at the HudsonAlpha Institute for Biotechnology, Jacob is leading a team that is finding ways to change people's lives. In his research, Jacob verifies that specific DNA changes cause disease, especially for undiagnosed conditions. And he wants to find a way to pinpoint those genetic conditions fast enough to benefit a patient. "Science is inherently slow and methodical, which is great," he says. "But not if you're trying to help a sick patient." Then Jacob adds his research to work from other HudsonAlpha investigators, bringing genome sequencing, data analysis and basic research together to make a diagnosis possible. "My role is to integrate these researchers that are doing fabulous work independently and create space for them to not only do world class science individually but also contribute to a larger team," Jacob says. "It's one part participant, one part coach, one part motivator." Jacob received his PhD in pharmacology from the University of Iowa in 1989. He completed his postdoctoral work at Harvard, Stanford and MIT. Before joining HudsonAlpha in 2015, Jacob was the founding director of the Human and Molecular Genetics Center and a professor in the departments of physiology and pediatrics at the Medical College of Wisconsin (MCW) in Milwaukee for nearly 20 years. At MCW, Jacob led a team that was the first in the world to successfully use DNA sequencing to identify and treat an unknown disease in a patient. That experienced saved the patient's life and changed Jacob's. "I always believed genomics was going to improve medicine," he says. "But it went from being a dream to being a passion. I'm frustrated that we're not helping more people today, when I know we could be changing lives. The good news is that we *are* going to be changing lives, and changing medicine, through genomics."

Stephen F. Kingsmore, MD, DSc - is President/CEO of the Rady Pediatric Genomic and Systems Medicine Institute at Rady Children's Hospital, San Diego, which is implementing pediatric genomic/precision medicine at unprecedented scale. Previously he was the Dee Lyons/Missouri Endowed Chair in Genomic Medicine at the Univ. of Missouri-Kansas City School of Medicine and Director of the Center for Pediatric Genomic Medicine at Children's Mercy Hospital, Kansas City. He has been the President/CEO of the National Center for Genome Resources, Santa Fe, New Mexico, Chief Operating Officer of Molecular Staging Inc., Vice President of Research at CuraGen Corporation, founder of GatorGen, and Assistant Professor at the University of Florida's School of Medicine. Dr. Kingsmore received MB ChB BAO and DSc degrees from the Queen's University of Belfast. He trained in clinical immunology in Northern Ireland and did residency in internal medicine and fellowship at Duke University Medical Center. He is a fellow of the Royal College of Pathologists. He was a MedScape Physician of the year in 2012, and received the 2013 Scripps Genomic Medicine award and 2013 ILCHUN prize of the Korean Society for Biochemistry and Molecular Biology. TIME magazine ranked his rapid genome diagnosis one of the top 10 medical breakthroughs of 2012.

Cecilia Lo, PhD – Dr. Cecilia Lo is Professor and Chair in the Department of Developmental Biology at the University of Pittsburgh School of Medicine. She is a developmental geneticist with special interest in the complex genetics of congenital heart disease. Her laboratory uses a systems genetic approach with mouse models to interrogate the genetic etiology of congenital heart disease (CHD). These studies utilize a unique integration of mouse fetal cardiovascular imaging with systems genetic analysis in large scale forward genetic screens that have led to new insights into the genomic context for CHD pathogenesis. In parallel, her laboratory is

engaged in human clinical studies that leverage genetic findings from the CHD mouse models to investigate the genetic etiology of human CHD. Findings from the CHD mouse models have also led to human clinical translational studies that have real potential for significant impact on improving the prognosis and standard of care for patients with CHD.

Cathleen Lutz, PhD, MBA – Dr. Lutz is the Repository Director and Research Scientist, Genetic Resources, The Jackson Laboratory. Dr. Lutz is head the Mouse Models Repository at The Jackson Laboratory (JAX), and is responsible for managing all aspects of the Repository which consists of over 1800 strains for distribution to the scientific community. Dr. Lutz is also a Senior Research Scientist at JAX, and works to develop innovative research tools to the worldwide scientific community. In this capacity, she engineers new models and conduct research in collaboration with many partners to speed the treatment of disease. Her concerted efforts in working with foundations and specialty areas within NIH led to the establishment in 2012 of the JAX Rare and Orphan Disease Center, which develops mouse models for rare diseases and works with researchers to refine and study new models with the aim to improve translational research from the mouse to the clinic. With a strong background in neuroscience, Dr. Lutz has recently expanded her translational interests to develop and standardize preclinical drug testing platforms for mouse models of neurological diseases. She runs the In Vivo Pharmacology Services in Bar Harbor and works with biotech and pharmaceutical companies in preclinical efficacy testing.

Daniel MacArthur, PhD - Daniel is a group leader within the Analytic and Translational Genetics Unit (ATGU) at Massachusetts General Hospital. He is also Assistant Professor at Harvard Medical School, and the Co-Director of Medical and Population Genetics at the Broad Institute of Harvard and MIT.

Calum MacRae, MD, PhD – Dr. MacRae is the Chief of Cardiovascular Medicine; Harvard Medical School, Brigham and Women's Hospital. Our lab is interested in the role of functional inputs such as mechanical forces, metabolism or electrical activity in refining the basic programs of cell specification and differentiation in cardiovascular development, disease, repair and regeneration. In a multidisciplinary approach to these problems, involving group members who are geneticists, developmental biologists, physiologists, computational biologists and engineers, we are working in three main areas. Unraveling the role of functional remodeling in the heart and vascular system - The basic cellular plan of the heart and vascular networks undergoes tremendous regional specialization during development. For example, gradients of myocyte function exist between the endocardium and epicardium, while each organ has a distinctive arterial or venous endothelium. Using the zebrafish and chick, we are combining classic developmental cell and molecular biology with high resolution in vivo cellular physiology to understand how genetic and epigenetic factors interact to generate this cellular diversity in conjunction with the genetic programs regulating organogenesis. Current projects include the generation of a functional fate map of the early heart, and dissecting the role of physiologic stimuli such as mechanical or electrical signals in the patterning of the heart and vasculature. Systems level disease modeling in the zebrafish – The zebrafish is uniquely positioned as a vertebrate model amenable to high-throughput screening. We have developed robust, automated in vivo assays that enable us to phenotype cellular or integrated cardiovascular function in 96 or 384 well plates. These tools facilitate quantitative, scalable approaches to disease pathway dissection in genetic models of human cardiac and vascular disorders. Genetic screens are underway in these disease models to identify novel pathway members and to explore gene-environment interactions, in particular pharmacogenetics. Parallel screens of small molecule libraries are designed to identify probes for chemical biology strategies in these same diseases with the ultimate aim of identifying new therapeutics. Genetics of human heart failure – We are also attempting to understand the genetics of common human cardiac and vascular disease. These syndromes have long been viewed as genetically "complex", but we have based our work on the premise that much of the apparent complexity reflects limited phenotypic resolution. In our human studies we are reevaluating disease phenotypes in classic kin-cohorts to resolve the genetic architecture of heart failure and vascular disease. These cohorts also enable investigation of early pathophysiologic mechanisms in 'preclinical' individuals (and in cells derived from these individuals), offering an avenue for the rapid translation of new diagnostic or therapeutic approaches.

As a physician and epidemiologist, **Teri Manolio, M.D., Ph.D.** has a deep interest in discovering genetic changes associated with diseases by conducting biomedical research on large groups of people. As the director of the new Division of Genomic Medicine, Dr. Manolio will lead efforts to support research translating those discoveries into diagnoses, preventive measures, treatments and prognoses of health conditions. "I see our division as a truly novel undertaking at NHGRI. We will take full advantage of the rapidly expanding knowledge base of genetic associations and of remarkable genome-scale analytic technologies," said Dr. Manolio. "This knowledge will enable us to begin to meld clinical and genomic research for rapid improvements in clinical care." Dr. Manolio envisions a day when patients have ready access to affordable, reliable genetic tests enabling them to avoid rare, sometimes devastating complications of common drug treatments. She also hopes to find ways of using a patient's genomic information to enhance diagnostic strategies and improve treatment outcomes by examining comprehensive databases of patients whose physical characteristics and genomic variants match those of the patient at hand. "Finding ways to achieve such goals through research will be an incredible challenge and I look forward to working with our new sister divisions, other National Institutes of Health (NIH) institutes and centers and the research community as a whole," Dr. Manolio said. Dr. Manolio joined NHGRI in 2005 as senior advisor to the NHGRI director for population genomics and as director of the Office of Population Genomics. She has led efforts to apply genomic technologies to population research, including theGenes, Environment, and Health Initiative (GEI), the Electronic Medical Records and Genomics (eMERGE) Network and the NHGRI Genome-Wide Association Catalog. Dr. Manolio came to NHGRI from NIH's National Heart, Lung, and Blood Institute where she was involved in large-scale cohort studies such as the Cardiovascular Health Study and the Framingham Heart Study. Dr. Manolio also maintains an active clinical appointment on the in-patient medical service of the Walter Reed National Military Medical Center, Bethesda, and is a professor of medicine on the faculty of the Uniformed Services University of the Health Sciences. She has authored more than 240 research papers and has research interests in genome-wide association studies of complex diseases and ethnic differences in disease risk. She received her B.S. in biochemistry from the University of Maryland College Park, her M.D. from the University of Maryland at Baltimore, and her Ph.D. in human genetics and genetic epidemiology from the Johns Hopkins School of Hygiene and Public Health.

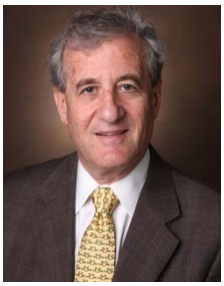
Howard McLeod, PharmD - Dr Howard McLeod is Medical Director of the DeBartolo Family Personalized Medicine Institute at the Moffitt Cancer Center. He is also a Senior Member of the Division of Population Sciences. Most recently he as the Fred Eshelman Distinguished Professor and Founding Director of the UNC Institute for Pharmacogenomics and Individualized Therapy, University of North Carolina, Chapel Hill. Dr McLeod held appointments in the UNC Schools of Pharmacy and Medicine, the Carolina Center for Genome Sciences, and the Lineberger Comprehensive Cancer Center. Dr McLeod is chair of the NHGRI eMERGE network external scientific panel and is a member of the FDA committee on Clinical Pharmacology. He is a member of the NIH NHGRI Advisory Council. Since 2002, Dr McLeod has been vice chair for Pharmacogenomics for the NCI clinical trials cooperative group CALGB/ALLIANCE, overseeing the largest oncology pharmacogenomics portfolio in the world. Dr McLeod is a 1000 talent scholar of China and a Professor at Central South University in Changsha, China. He also directs the Pharmacogenetics for Every Nation Initiative, which aims to help developing countries use genetic information to improve National Drug Formulary decisions. Howard has published over 460 peer reviewed papers on pharmacogenomics, applied therapeutics, or clinical pharmacology and continues to work to integrate genetics principles into clinical practice to advance individualized medicine.

Robert Nussbaum, MD - Dr. Nussbaum is currently the Chief Medical Officer of Invitae, a genetic information and testing company. From 2006-2015, he was the Holly Smith Professor of Medicine at UCSF, Chief of the Division of Genomic Medicine, and led both the Cancer Risk Program at the Helen Diller Family Cancer Center and the UCSF Program in Cardiovascular Genetics. He was a member of the Executive Committee of the UCSF Institute for Human Genetics and directed the recently formed Genomic Medicine Initiative at UCSF. He came to UCSF in 2006 from the Division of Intramural Research of the National Human Genome Research Institute, NIH, where he served for 12 years as Chief of the Genetic Diseases Research and Inherited Disease Research Branches. He is board certified in internal medicine, clinical genetics and clinical molecular genetics. He is the co-author of over 200 peer-reviewed publications in human genetics and co-author with Drs. Roderick M. McInnes and Huntington F. Willard of the popular textbook of human genetics, Thompson and Thompson's Genetics in Medicine. Dr. Nussbaum is a

Director and Treasurer of the American Board of Medical Genetics and Genomics and has served on the Board of Directors of the American College of Medical Genetics and Genomics and on the Board of the American Society of Human Genetics, where he also served as President. He was elected to the Institute of Medicine in 2004 and the American Academy of Arts and Sciences in 2015.

Jose Ordovas, PhD – Jose M. Ordovas, PhD, is Professor of Nutrition and a Senior Scientist at the USDA-Human Nutrition Research Center on Aging at Tufts University in Boston where he also is the Director of the Nutrition and Genomics Laboratory. He is also a Senior Scientist at the CNIC, and at IMDEA-FOOD both in Madrid. Dr. Ordovas was educated in Spain at the University of Zaragoza where he completed his undergraduate work in chemistry (1978) and received his doctorate (1982). He did postdoctoral work at the MIT, Harvard and Tufts. Dr. Ordovas' major research interests focus on the genetic factors predisposing to cardiovascular disease and obesity and their interaction with the environment and behavioral factors with special emphasis on diet. More recently he has included chronobiology as part of the analysis of gene by environment interactions. He has published more than 700 scientific articles and written numerous reviews and books on these topics. In this regard, he is considered one of the most distinguished world experts in gene-diet interactions related to cardiovascular traits. Moreover, he has trained in his laboratory over 60 scientists and his current genetic studies involve populations and investigators from all Continents. Throughout his career, Dr. Ordovas has received multiple honors for his scientific achievements including, among many others, the USDA Secretary's Award, and the Danone Foundation Award for achievements in Nutrition Research. He holds an honorary degree in Medicine by the University of Cordoba (Spain) and he is member of the Spanish Royal Academies of Sciences, Medicine, Nutrition and Pharmacy.

Peter Robinson, PhD – Associate Professor, Charite Univeritatsmedizin Berlin; Institute of Medical Genetics and Human Genetics. Peter N. Robinson is a research scientist and leader of the Computational Biology Group in the Institute of Medical Genetics and Human Genetics at Charité-Universitätsmedizin Berlin. Dr. Robinson completed his medical education at the University of Pennsylvania, followed by an internship at Yale University. He also studied mathematics and computer science at Columbia University. His research interests involve the use of mathematical and bioinformatics models to understand biology and hereditary disease.



Dan Roden, MD - Dan Roden received his medical degree and training in Internal Medicine from McGill University in Montreal, and trained in Clinical Pharmacology and Cardiology at Vanderbilt, where he has been a faculty member since. His initial career focus – that he has maintained – was studies of the clinical, genetic, cellular, and molecular basis of arrhythmia susceptibility and variability responses to arrhythmia therapies. Over the last 10 years, Dr. Roden has led Vanderbilt's broader efforts in pharmacogenomics discovery and implementation. He is principal investigator for the Vanderbilt sites of the National Institutes of Health's Pharmacogenomics Research Network (PGRN) and the NHGRI's Electronic Medical Records and Genomics (eMERGE) Network. He directs the Vanderbilt DNA databank BioVU, a discovery resource that as of December 2013 included >174,000 samples linked to deidentified electronic medical records. He is a leader in the PREDICT project that since 2010 has been preemptively embedded pharmacogenomic variant data in the electronic medical records of >14,000 Vanderbilt patients; PREDICT currently displays information on 5 drug-gene pairs and delivers point of care decision support when a target drug is prescribed to patients with variant genotypes. Dr. Roden has received the Leon Goldberg Young Investigator Award and the Rawls Palmer Progress in Science Award from the American Society for Clinical Pharmacology and Therapeutics; the Distinguished Scientist Award and the Douglas Zipes lectureship from the Heart Rhythm Society; and the Distinguished Scientist award from the American Heart Association. He currently serves on the Science Board of the FDA. He has been elected to membership in the American Society for Clinical Investigation and the Association of American Physicians, and fellowship in the American Association for the Advancement of Science.

Laura Lyman Rodriguez, Ph.D., is the Director of the Division of Policy, Communications, and Education. In this capacity, she works to develop and implement policy for research initiatives at the NHGRI, design communication and

outreach strategies to engage the public in genomic science, and prepare health care professionals for the integration of genomic medicine into clinical care. Dr. Rodriguez is particularly interested in the policy and ethics questions related to the inclusion of human research participants in genomics and genetics research. As a major contributor to the development of the National Institutes of Health (NIH) Policy for Data Sharing in Genome-Wide Association Studies (GWAS), Dr. Rodriguez assisted in shaping the policy for sharing NIH-supported genomic research data. She has also played a significant role in establishing and overseeing implementation of the genomic data sharing policy across the agency. Dr. Rodriguez received her bachelor's degree with honors in biology from Washington and Lee University in Lexington, Va. and earned a doctorate in cell biology at Baylor College of Medicine in Houston. She served as administrative director at the Baylor Institute for Immunology Research after graduating in 1996, and it was through this work — helping to establish that institute and its laboratories — that Dr. Rodriguez became interested in clinical research policy. Dr. Rodriguez began her policy career as a Congressional Science Fellow with the American Association for the Advancement of Science (AAAS), where she worked with Rep. Vernon J. Ehlers (R-Mich.) on his National Science Policy Report and K-12 math and science education issues. Dr. Rodriguez also spent time in the Office of Public Affairs at the Federation of American Societies for Experimental Biology, where she focused on legislative relations. Before joining NHGRI in late 2002, she spent time at the National Academies, Institute of Medicine where she directed the work of a committee examining the federal system for protecting human research participants that produced two reports:*Preserving Public Trust: Accreditation, and Human Research Participant Protection Programs and Responsible Research: A Systems Approach to Protecting Research Participants*. In addition to her policy work at the NHGRI, Dr. Rodriguez served as the Special Advisor to the Director from 2003 through 2007. During this time she provided scientific support and coordination for the activities of the director and helped to design and implement a new administrative structure for the Immediate Office of the Director. In 2008, she was named the Senior Advisor to the Director for Research Policy and was later appointed to be the Deputy Director for OPCE.

Wendy Rubinstein, MD, PhD - DIRECT NIH GENETIC TESTING REGISTRY (GTR), Directed the final development and launch of the world's first free, centralized online resource for information about the availability and scientific basis of genetic tests. GTR has over 32,000 detailed tests registered by laboratories in 40 countries for over 6,000 conditions. Support development and adoption of genetic testing standards – variant interpretation, molecular test codes, phenotype and variant nomenclatures, reference materials for NGS. Engage diverse stakeholders – testing laboratories and manufacturers, pharmaceutical companies, medical professional societies, hospital organizations, information management organizations. Augment clinical decision support (CDS) at point of care (POC) via electronic health records (EHR/EMR) using contextual information via HL7 Infobutton messaging for NCBI's resources including GTR, ClinVar, and MedGen's professional practice guidelines. NIH Director's Award, GTR Policy Team (2012). LIAISON, NIH OFFICE OF THE DIRECTOR - Liaison to FDA, CMS, CDC, and other federal agencies to support activities surrounding genetic testing such as LDT guidance, PAMA, CLIA, NGS database. Provide strategic analysis and proposals to support federal oversight. Introduce President's Precision Medicine Initiative to the laboratory industry (e.g., California Clinical Laboratory Association 2015 annual conference) Institute of Medicine (IOM) Genomics Roundtable and DIGITizE Action Collaborative. Work with domestic and international organizations and other HHS agencies on policy issues. AMA, AMP, ACLA, ACS, ASCO, ASHG, ACMG, CAP, CPIC, PharmGKB, McKesson, Palmetto, NCI, NSGC, NIST/GIAB, Genetic Alliance, BSBSA, MSKCC, Dana Farber Cancer Institute, MD Anderson Cancer Center, Kaiser Permanente, UCSF, Baylor, Geisinger, GA4GH, ENIGMA, CIMBA, InSiGHT, EPIC, Cerner, HL7, etc.

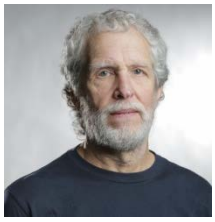
Christine (Kricket) Seidman, MD - Christine Seidman, MD is the Thomas W. Smith Professor of Medicine and Genetics at Harvard Medical School and Brigham and Women's Hospital and an Investigator of the Howard Hughes Medical Institute. She was an undergraduate at Harvard College and received a M.D. from George Washington University School of Medicine. After clinical training in Internal Medicine at John Hopkins Hospital she received subspecialty training in cardiology at the Massachusetts General Hospital. Dr. Seidman is a faculty member of Brigham and Women's Hospital, where she serves as Director of the Brigham Research Institute. She is the founding Director of the BWH Cardiovascular Genetics Center. Dr. Seidman's laboratory uses genomic strategies to define causes of human cardiovascular disease, including congenital heart malformations and cardiomyopathies. By exploiting model systems to identify pathways

impacted by mutations, these studies have enabled gene-based diagnostics and novel strategies to limit the deleterious consequences of human mutations. Dr. Seidman also leads multi-institution consortium that assess rare and common variants involved in cardiovascular phenotypes and that explore the clinical utility of genomic variation in early diagnosis and prevention of cardiovascular disease. The recipient of many honors, Dr. Seidman is a Distinguished Scientist of the American Heart Association, Fellow of the American Academy of Arts and Sciences, and member of the Institutes of Medicine and the National Academy of Sciences. She is also President of the Association of American Physicians.

Lea Starita, PhD - I completed my graduate work on the biochemistry of the BRCA1 protein in the lab of Jeff Parvin at Harvard University. I then went on to postdoc with Stan Fields, where my work on ubiquitin ligases brought me back to BRCA1. I am currently a senior scientist with Jay Shendure in the Genome Sciences department at the University of Washington where I develop high throughput methods to determine the effects of mutations on protein function.

Barbara Stranger, PhD - Dr. Barbara Stranger is an Assistant Professor of Medicine in the Section of Genetic Medicine at the University of Chicago. She is also a Core Member of the Institute for Genomics and Systems Biology and a Senior Fellow in the Center for Data Intensive Science. She has a longstanding interest in understanding how genetic variation influences complex human traits in humans, with focus on genetic effects on intermediate molecular traits such as gene expression. Her lab collects and analyzes large scale multi-dimensional human genomics data, particularly transcriptome data and genetic variation data, in the context of health and disease. Her lab is interested in how context influences the association between genetic variation and trait variation. The lab has projects investigating the effects of cell and tissue type, cellular stimulation status, disease status, age, and sex on trait variation. Her lab is part the Genotype-Tissue Expression (GTEx) Consortium, both as data generators and members of the Analysis Working Group (AWG), and Barbara leads the gender subgroup of GTEx AWG.

Haoyi Wang, PhD - Dr. Wang is an expert in the filed of genome engineering. The Wang lab focuses on developing novel technologies to achieve more efficient and specific genome and epigenome editing. Dr. Wang has an interdisciplinary training in genetics, molecular biology, and stem cell biology that makes him uniquely positioned to explore this area. As a doctoral and post-doctoral researcher, Dr. Wang has worked on the development of a variety of genome engineering technologies, including transposon-based "Calling Card" method for determining the genome-wide binding locations of transcription factors, TALEN-mediated genome editing in human pluripotent stem cells and mice, CRISPR-mediated multiplexed genome editing in mice, and CRISPR-mediated gene activation in human cells. Since he joined JAX in early 2014, his lab has developed novel method for delivering CRISPR reagent into zygotes, which significantly improved the efficiency of mouse model generation. In addition to continually improving TALEN and CRISPR technologies to achieve better efficiency and specificity, Wang lab will also explore novel strategies and applications for genome and epigenome editing.



Monte Westerfield, PhD – is a Professor in the Department of Biology at the Institute of Neuroscience at the University of Oregon. Molecular genetic basis of human diseases; ear and eye development and pathogenesis.

Overview: Our laboratory studies the molecular genetic basis of human diseases, particularly Usher syndrome, the leading cause of combined deafness and blindness, and other diseases of the eye and ear. We use zebrafish and a combination of anatomical, physiological, molecular, and genetic techniques. The goal of our research is to identify disease-causing genes, to elucidate what goes wrong during disease, and to develop preclinical trials for new therapies. Current research focuses on developing models of human disease. In particular, we are making and studying models of Usher syndrome. Our research is funded by the National Eye Institute, the National Institute on Deafness and Other Communicative Disorders, the National Institute of Child Health and Development, and the Office of the Director of the National Institutes of Health. We also thank friends and family of Jodi Carter and Ryan Thomason and The Megan Foundation for generous donations to the Usher Syndrome Research Fund that supports our work.

Marc S. Williams, MD, FAAP, FACMG is an alumnus of the University of Wisconsin-Madison having graduated with a BS in Chemistry in 1977, and an MD in 1981. He did a pediatric residency at the University of Utah from 1981-1984. After two years of solo practice in Hillsdale, Michigan, he joined the Riverside (California) Medical Clinic as a general pediatrician and practiced there until 1991. From 1991 until joining Intermountain Healthcare, Dr. Williams was at the Gundersen Lutheran Medical Center in La Crosse, WI. Hired as a general pediatrician, he eventually pursued fellowship training in Clinical Genetics, and was board certified in this specialty in 1996 and recertified in 2006. In 1999, he gave up general pediatric practice and became the associate medical director of the Gundersen Lutheran Health Plan while maintaining his genetic practice. It was by combining these two areas of expertise that he developed an interest in the role of genetics in health care delivery. He has published and presented extensively on this topic. From January of 2005 to December 2011 he was the director of the Intermountain Healthcare Clinical Genetics Institute in Salt Lake City, Utah. As of January 2012, he has been the director of the Genomic Medicine Institute of the Geisinger Health System in Danville, Pennsylvania. He is a director of the board of the American College of Medical Genetics and in 2009 was elected Vice-President of Clinical Genetics of the College. He has participated in the Personalized Medicine Workgroup of the Department of Health and Human Services' American Health Information Community Task Force, chaired the CDC's EGAPP Stakeholder's Group, was a member of the CDC's CETT program review board and the Secretary's Advisory Committee for Genetics, Health and Society, having previously served on the Coverage and Reimbursement Task Force of that group. He was recently appointed to the EGAPP working group. He is past chair of the Committee on the Economics of Genetic Services of the American College of Medical Genetics, as well as chair of the subcommittee on Health Care Systems of the Section on Genetics and Birth Defects of the American Academy of Pediatrics. He is currently chairing the ACMG Ad Hoc Committee on the Value of a Genetic Diagnosis. He founded the American College of Medical Genetics Quality Improvement Special Interest Group. He is the Editor-in-Chief of the Manual on Reimbursement for Medical Genetic Services. He has authored over fifty articles in the peer-review medical literature and is a frequent presenter at national and international meetings.

Elizabeth Worthey, PhD - Liz Worthey is the Director of Software Development and Informatics at HudsonAlpha Institute for Biotechnology with close to 20 years experience in the fields of human disease, human genetics, molecular genetics, bioinformatics, and translational and clinical genomics. The central research theme across her career has been application of cutting edge technologies to identify and study potential differentiators in disease induction, progression, outcome, and treatment including identification of novel therapeutics. She has been involved in developing novel methods and tools to study how molecular alterations contribute to basic biology, host-human pathogen interaction, and human diseases; pediatric and adult cancer, ocular, vascular, neurologic, pulmonary, developmental, and immune disorders. She was among the first to apply genome wide sequencing as a clinical test and together with colleagues build the first genomics based genetics clinic. For years she has overseen the development of methods and tools for clinical diagnostic genome wide sequencing to identify causative mutations and has been a PI on a number of projects within this field including through Keck Foundation funding and within the NIH Undiagnosed Disease Network. Worthey received her Ph.D. in Genetics from the University of London in 2003 and completed her postdoctoral fellowship at the Seattle Biomedical Research Institute at the University of Washington, working on both Eukaryotic Genomics and other high throughput "Omics" projects. In 2008, after working as a Project Manager for Merck & Co., Worthey joined the Medical College of Wisconsin (MCW) as a Senior Research Scientist and became an Assistant Professor of Pediatric Genomics in 2010 and Director of Genomic Informatics for the Human and Molecular Genetics Center in 2012. In 2015 Worthey joined the HudsonAlpha Institute for Biotechnology as a Faculty Investigator and Director of Genomic Informatics.

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METRORAIL

Each passenger needs a farecard to enter and exit. Up to two children under age five may travel free with a paying customer.

Fares are based on when and how far you ride. Station-to-station fares are located on station manager kiosks and FARECARDS & PASSES machines. The machines accept credit and debit cards. If you use cash, bring small bills; the machines only provide up to $10 in change (in coins).

Save money by using a SmarTrip® card instead of a paper farecard. SmarTrip® is a plastic farecard that can be reloaded with value and reused to pay Metrorail and Metrobus fares and parking fees at Metro-operated lots.

SmarTrip® cards are sold from dispensing machines at Metrorail stations. You can also buy a SmarTrip® card at over 200 retail locations or online at wmata.com. You can add value to your SmarTrip® card at FARECARDS & PASSES machines in Metrorail stations, at select retail locations and on our website. You can also check your registered card's value, see where and when you used it last or report it lost or stolen on our website.

If you plan to ride a lot in one day, get one day of unlimited Metrorail rides with a One Day Pass. Buy it from a FARECARDS & PASSES machine in Metro stations.

HOURS OF SERVICE

Open:	5 a.m. Mon.-Fri.	7 a.m. Sat.-Sun.
Close:	midnight Sun.-Thurs.	3 a.m. Fri.-Sat. nights

Last train times vary. To avoid missing the last train, please check the last train times posted in stations.

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Senior citizens 65 and older and persons with disabilities may ride for half the regular fare. On Metrorail, use a senior or disabled SmarTrip® card or a SmarTrip-enabled WMATA-issued disability ID. On Metrobus, use a senior or disabled SmarTrip® card or a Metro Disability ID. Customers can also present a valid Medicare card and a photo ID and pay the reduced fare in cash. For details about qualifying for a Metro ID and buying senior or disabled SmarTrip® cards, visit wmata.com or call 202-637-7000 (TTY 202-638-3780).

Free orientations on how to use accessible Metrobus and Metrorail services are available to senior citizens and people with disabilities by calling 202-962-1100 (TTY 202-962-2033).

PARKING AT METRO STATIONS

There is a daily fee to park at Metro lots and garages Monday through 3 a.m. Saturday morning. There is no parking fee on weekends and federal holidays. Cash is not accepted, except at metered spaces. All Metro lots and garages accept payment with a SmarTrip® card and most accept major credit cards. Daily parking fees vary by station and are posted at the parking entrance/exit and on our website.

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To avoid long lines at farecard machines after a major event, make sure you have roundtrip fare on your SmarTrip® card (or buy a roundtrip farecard) at the start of your trip.

The weekday rush-hour periods — before 9:30 a.m. and between 3 and 7 p.m. — are the most crowded. If possible, plan your trip to avoid those times.

If you lose an item on a bus or train or in a station, please call Lost & Found at 202-962-1195 or visit our website.

Sign up for MetroAlerts at wmata.com and get timely, targeted Metro information sent right to your desktop or mobile device.

Information subject to change. 50.532 (REV. 4/14)

Metrorail Pocket Guide

RIDER INFORMATION
METRO SYSTEM MAP
POINTS OF INTEREST

RD OR BL GR YL SV

wmata.com

202-637-7000 — TTY 202-638-3780

POINTS OF INTEREST

- African Art Museum ... Smithsonian
- Air and Space Museum ... L'Enfant Plaza
- American Art Museum ... Gallery Place
- American History Museum ... Smithsonian
- Botanic Gardens ... Federal Center SW
- Bureau of Engraving & Printing ... Smithsonian
- Capitol Building ... Capitol South
- Chinatown ... Gallery Place
- Constitution Hall ... Farragut West
- Convention Center ... Mt Vernon Sq
- Corcoran Gallery ... Farragut West
- D.C. Armory ... Stadium-Armory
- FedEx Field ... Morgan Boulevard
- Federal Bureau of Investigation ... Metro Center
- Folger Shakespeare Library ... Capitol South
- Freer Gallery ... Smithsonian
- Hirshhorn Museum ... L'Enfant Plaza
- Holocaust Museum ... Smithsonian
- House Where Lincoln Died ... Metro Center
- Iwo Jima Memorial ... Rosslyn
- Jefferson Memorial ... Smithsonian
- Library of Congress ... Capitol South
- Lincoln Memorial ... Foggy Bottom-GWU
- Lisner Auditorium ... Foggy Bottom-GWU
- Martin Luther King Jr. Library ... Gallery Place
- Martin Luther King Jr. Mem'l ... Smithsonian
- Masonic Temple ... King Street-Old Town
- Nat'l Academy of Sciences ... Foggy Bottom-GWU
- National Aquarium ... Federal Triangle
- National Archives ... Archives
- National Building Museum ... Judiciary Sq
- National Gallery of Art ... Archives
- National Geographic Society ... Farragut North
- National Institutes of Health ... Medical Center
- National Museum of the American Indian ... L'Enfant Plaza
- National Portrait Gallery ... Gallery Place
- National Postal Museum ... Union Station
- National Shrine of the Immaculate Conception ... Brookland-CUA
- National WWII Memorial ... Smithsonian
- National Zoo ... Woodley Park
- Nationals Ballpark ... Navy Yard-Ballpark
- Natural History Museum ... Smithsonian
- Phillips Collection ... Dupont Circle
- Renwick Gallery ... Farragut West
- RFK Stadium ... Stadium-Armory
- Sackler Gallery ... Smithsonian
- Smithsonian Castle ... Smithsonian
- Spy Museum ... Gallery Place
- State Department ... Foggy Bottom-GWU
- Supreme Court ... Capitol South
- Verizon Center ... Gallery Place
- Vietnam Veterans Memorials ... Foggy Bottom-GWU
- Washington Monument ... Smithsonian
- Washington National Cathedral[1] ... Tenleytown-AU
- White House and Visitor Center ... Federal Triangle

COLLEGES AND UNIVERSITIES

- American Univ.[2] ... Tenleytown-AU
- Catholic Univ. of America ... Brookland-CUA
- Gallaudet Univ. ... NoMa-Gallaudet U
- George Mason Univ. ... Vienna and Virginia Sq-GMU
- Georgetown University ... Foggy Bottom-GWU
- George Washington Univ. ... Foggy Bottom-GWU
- Howard Univ. ... Shaw-Howard U
- Johns Hopkins Univ. ... Dupont Circle
- Marymount Univ. ... Ballston-MU
- Marymount Univ./Reston ... Wiehle-Reston East
- No. Va. Comm. Coll./Reston ... Wiehle-Reston East
- Univ. of the District of Columbia ... Van Ness-UDC
- Univ. of Maryland[2] ... College Park-U of Md
- Va. Tech./UVA No. Virginia Center ... West Falls Church

SHOPPING

- Ballston Common ... Ballston–MU
- Connecticut Avenue ... Farragut North
- City Place ... Silver Spring
- Crystal City Underground ... Crystal City
- Fashion Centre ... Pentagon City
- Georgetown[3] ... Foggy Bottom-GWU
- Mazza Gallerie/Chevy Chase Pavilion/Chevy Chase ... Friendship Heights
- Old Post Office Pavilion ... Federal Triangle
- Plaza America ... Wiehle-Reston East
- Shops at National Place ... Metro Center
- The Mall At Prince Georges ... Prince Georges Plaza
- Tysons Corner Center ... Tysons Corner
- Tysons Galleria ... Tysons Corner
- Wheaton Plaza ... Wheaton

THEATERS

- Arena Stage ... Waterfront
- Discovery Theatre ... Smithsonian
- Folger Theatre ... Capitol South
- Ford's Theatre ... Metro Center
- Kennedy Center[4] ... Foggy Bottom-GWU
- Lincoln Theatre ... U Street
- National Theater ... Metro Center
- Shakespeare Theater ... Archives
- Strathmore ... Grosvenor-Strathmore
- Warner Theatre ... Metro Center
- Wolf Trap[5] ... West Falls Church
- Woolly Mammoth Theatre Co ... Archives

[1] From this station, transfer to any southbound 30-series Metrobus.
[2] University-operated shuttle between station and campus.
[3] Georgetown Metro Connection shuttle runs through Georgetown from Foggy Bottom-GWU station (also Rosslyn and Dupont Circle stations).
[4] Kennedy Center shuttle between station and Center.
[5] Wolf Trap shuttle between station and park during season.

System Map

wmata.com
Customer Information Service: 202 637-7000
TTY Phone: 202 638-3780
Metro Transit Police: 202 962-2121

Legend

- RD Red Line • Glenmont / Shady Grove
- OR Orange Line • New Carrollton / Vienna
- BL Blue Line • Franconia-Springfield / Largo Town Center
- GR Green Line • Branch Ave / Greenbelt
- YL Yellow Line • Huntington / Fort Totten
- SV Silver Line • Wiehle-Reston East / Largo Town Center

Station Features
- Bus to Airport
- Parking
- Hospital
- Airport

Connecting Rail Systems: Amtrak, VRE, MARC

Transfer Station
Under Construction
Full-Time Service
Station in Service

Rush-Only Service: Monday-Friday
6:30am - 9:00am 3:30pm - 6:00pm

- No Smoking
- No Eating or Drinking
- No Animals (except service animals)
- No Audio (without earphones)
- No Littering or Spitting
- No Dangerous or Flammable Items

from Baltimore/Washington International Thurgood ...
to Sheraton Silver Spring Hotel, 8777 Georgia Avenue,...

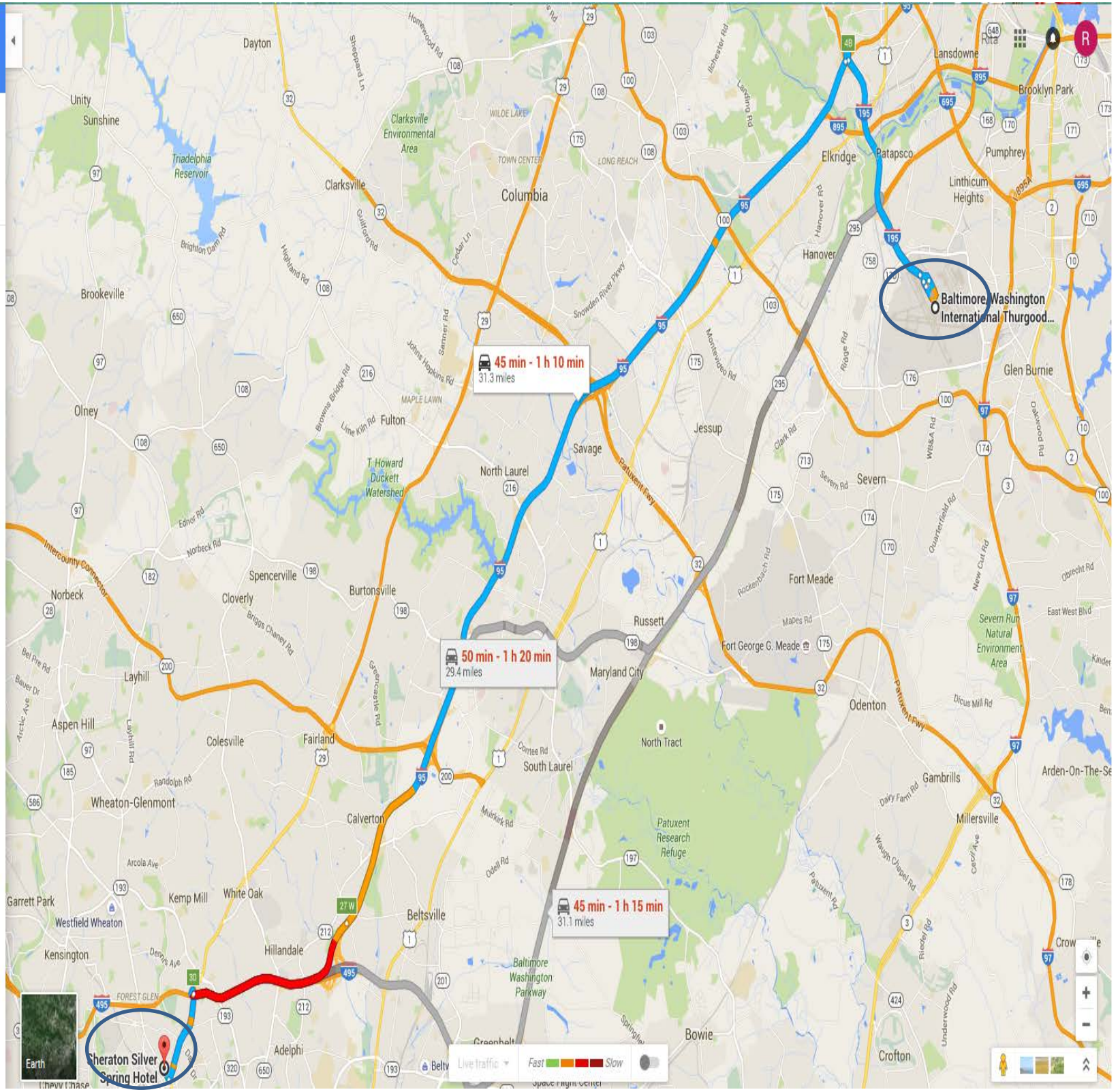
45 min - 1 h 10 min
(31.3 miles)
via I-95 S

Baltimore/Washington International Thurgood Marshall Airport
Baltimore, MD 21240

1. Follow Service Rd Lower Level and Friendship Rd to I-195 W
   4 min (1.0 mi)
2. Take I-95 S to US-29 S/Colesville Rd/Columbia Pike in Silver Spring. Take exit 30 from I-495 W
   28 min (28.5 mi)
3. Continue on US-29 S/Colesville Rd. Drive to Georgia Ave
   6 min (1.8 mi)

Sheraton Silver Spring Hotel
8777 Georgia Avenue, Silver Spring, MD 20910

These directions are for planning purposes only. You may find that construction projects, traffic, weather, or other events may cause conditions to differ from the map results, and you should plan your route accordingly. You must obey all signs or notices regarding your route.



from Ronald Reagan Washington National Airport, Arli...
to Sheraton Silver Spring Hotel, 8777 Georgia Avenue,...

30 - 40 min (24.8 miles)

via George Washington Memorial Pkwy and I-495 N

Ronald Reagan Washington National Airport
Arlington, VA 22202

- Get on George Washington Memorial Pkwy
  33 s (0.2 mi)
- Follow George Washington Memorial Pkwy and I-495 N to MD-97 S/Georgia Ave in Silver Spring. Take exit 31B from I-495 N
  28 min (23.4 mi)
- Drive to Georgia Ave
  5 min (1.2 mi)

Sheraton Silver Spring Hotel
8777 Georgia Avenue, Silver Spring, MD 20910

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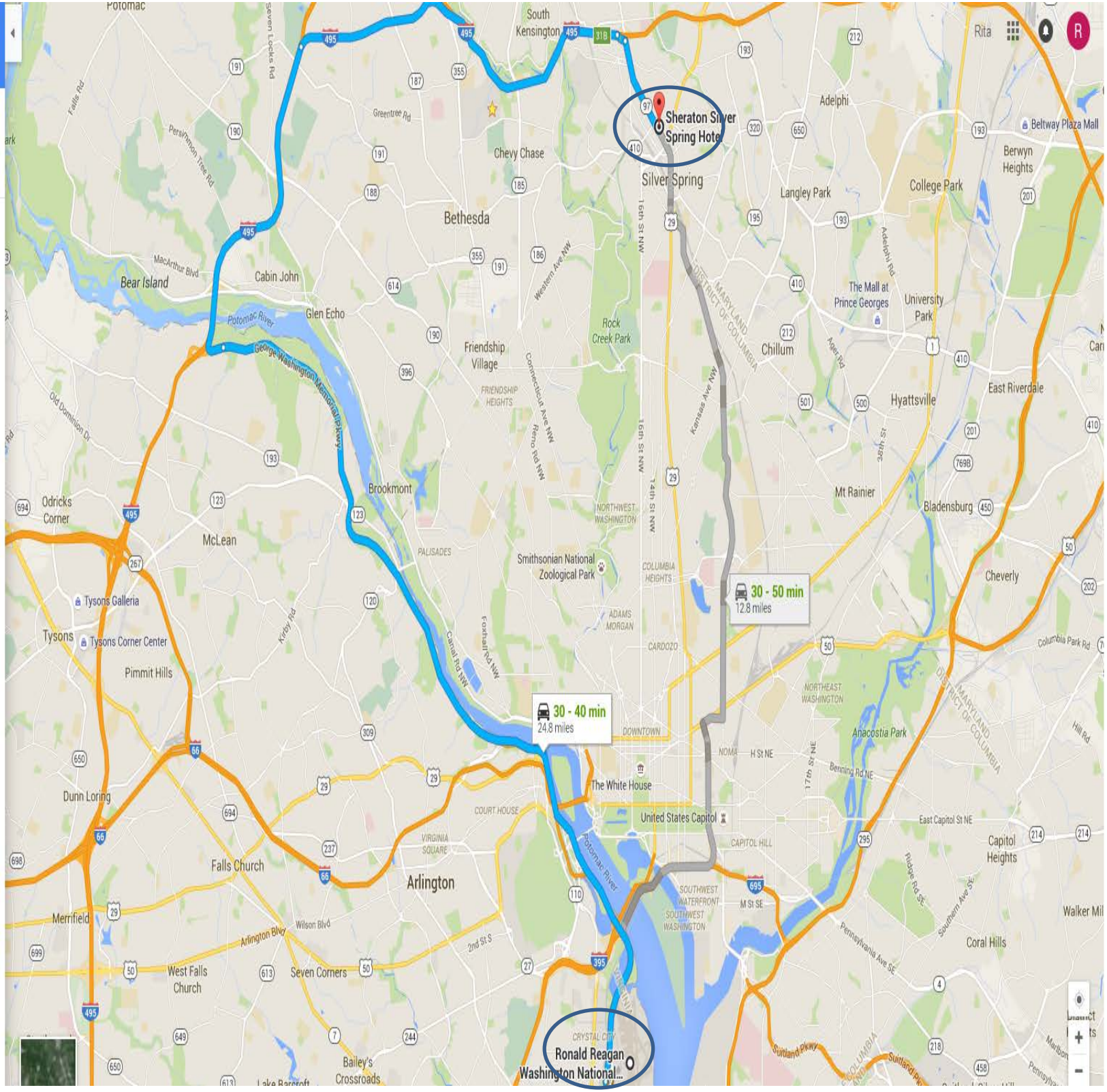


from Dulles International Airport, 1 Saarinen Circle, Du...
to Sheraton Silver Spring Hotel, 8777 Georgia Avenue,...

35 - 45 min (29.3 miles)

via Dulles Access Rd and I-495 N

Dulles International Airport

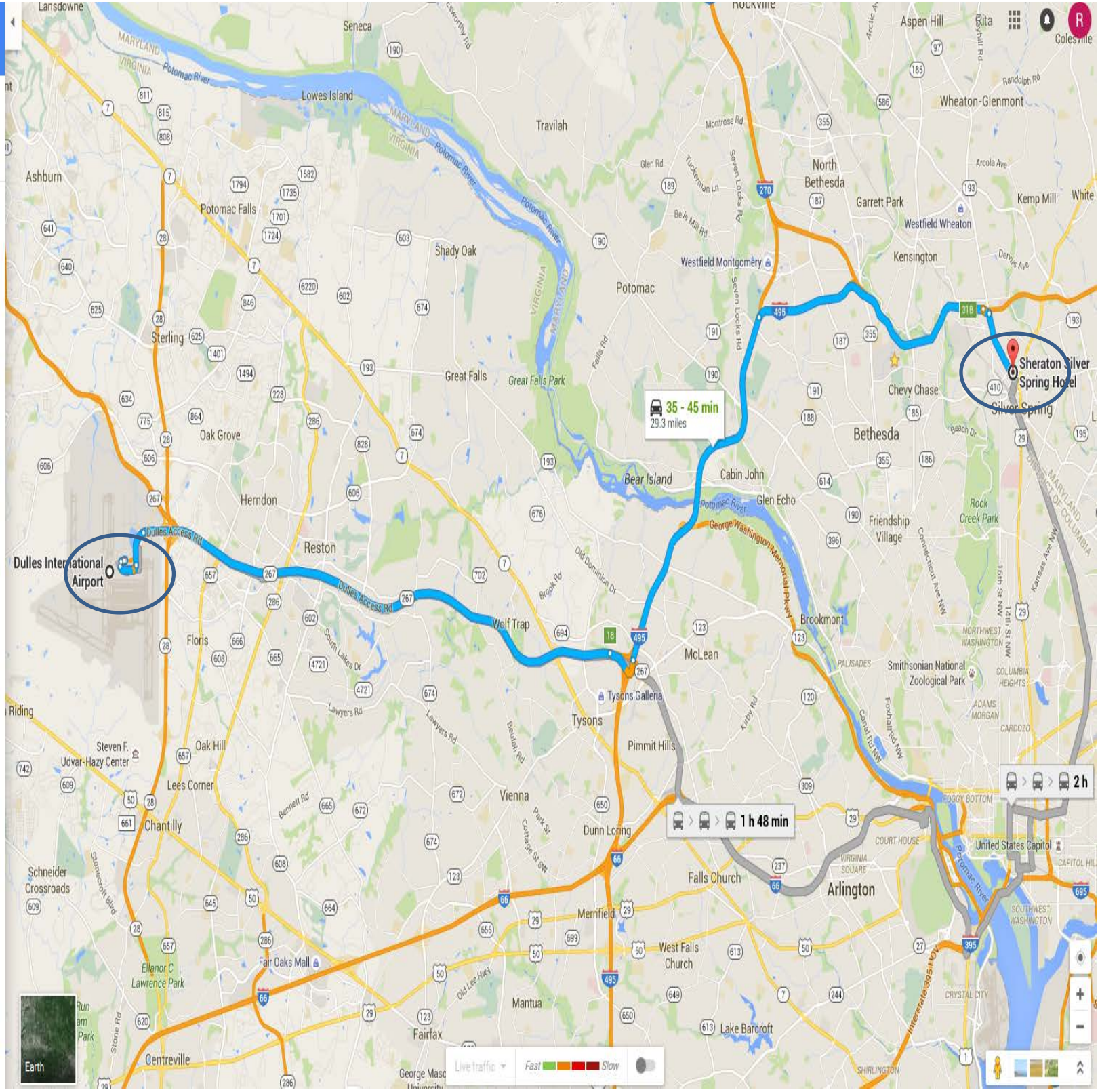
1 Saarinen Circle, Dulles, VA 20166

- Follow Saarinen Cir to Dulles Access Rd
  - 2 min (0.7 mi)
- Follow Dulles Access Rd and I-495 N to MD-97 S/Georgia Ave in Silver Spring. Take exit 31B from I-495 N
  - 30 min (27.4 mi)
- Drive to Georgia Ave
  - 5 min (1.2 mi)

Sheraton Silver Spring Hotel

8777 Georgia Avenue, Silver Spring, MD 20910

These directions are for planning purposes only. You may find that construction projects, traffic, weather, or other events may cause conditions to differ from the map results, and you should plan your route accordingly. You must obey all signs or notices regarding your route.



Genomic Medicine IX:
NHGRI's Genomic Medicine Portfolio

April 19-20, 2016

Sheraton Silver Spring
8777 Georgia Avenue
Silver Spring, MD 20910
(301) 468-1100
www.sheratonsilverspring.com

DRIVING DIRECTIONS

Sheraton Silver Spring Hotel

8777 Georgia Avenue
Silver Spring, MD 20910
United States
Phone 1-301-589-0800

General Directions to the Hotel

From the Washington Beltway, Interstate 495

- Take Exit 31, Georgia Avenue South.
- Proceed approximately 1 mile.
- The hotel is ahead on the left and is the first high-rise upon entering downtown Silver Spring.

From Ronald Reagan National Airport (DCA)

- Take George Washington Memorial Parkway.
- Merge onto Interstate 395 north toward Washington.
- Take US 1 North to Exit 1.
- Stay straight to 14th Street NW.
- Turn right onto New York Avenue.
- Stay straight onto K Street NW.
- Turn left onto 7th Street NW.
- 7th Street NW becomes Georgia Avenue.
- The hotel is on the right.

From South

- Follow Interstate 95 North.
- Take Exit 170B for Interstate 495 North towards Tysons Corner (inner loop of the beltway).
- Merge on to Interstate 495.
- Take Exit 31B, Georgia Avenue South.
- The hotel is ahead on the left and is the first high-rise upon entering downtown Silver Spring.

Genomic Medicine IX:
NHGRI's Genomic Medicine Portfolio

April 19-20, 2016

Sheraton Silver Spring
8777 Georgia Avenue
Silver Spring, MD 20910
(301) 468-1100
www.sheratonsilverspring.com

From BWI Airport

- Take Interstate 195 West.
- Exit onto Interstate 95 South.
- Take Exit 27 for Interstate 495 West towards Silver Spring.
- Merge on Interstate 495 (outer loop).
- Take Exit 31, Georgia Avenue South.
- The hotel is ahead on the left and is the first high-rise upon entering downtown Silver Spring.

From North

- Take Interstate 95 South towards Washington D.C.
- Take Exit 27 for Interstate 495 West towards Silver Spring.
- Merge onto Interstate 495.
- Take Exit 31, Georgia Avenue South.
- The hotel is ahead on the left and is the first high-rise upon entering downtown Silver Spring.

http://www.opentable.com/s/?covers=2&datetime=2016-04-19%2019:00&excludefields=Description&metroid=9&neighborhoodids=495,808&regionids=188&showmap=true&size=100&currentview=map

Genomic Medicine IX Support Team
April 19-20, 2016
Silver Spring, MD

Rita L. Chambers, BS - is the Senior Program Coordinator of the Center for Applied Genomic & Precision Medicine; at Duke University for over the past nine years. She is also the support team for the NHGRI Genomic Medicine Meetings. Ms. Chambers has over 20 years' combined experience in the administration, organization, and promoting meetings and activities for numerous organizations and corporations including Duke University, LabCorp, and Segro-Colonial Abrasives. She has been acknowledged for her efforts with the organization of "*Genomic and Personalized Medicine*", Second Edition, V1-2 Hardcover, Ginsburg GS and Willard HW, 2012. Organized and Editorial Efforts in keeping the project on task for completion. She was also awarded the Presidential Meritorious Award for her recognition of outstanding services to Duke University. Ms. Chambers has a background in Business Administration with a concentration in Management and Organizational Development and currently studying for her Dual Master's Degrees in Business Administration and Science in Leadership at Pfeiffer University in Charlotte, NC.

Elian Silverman, BA – the Scientific Program Analyst for the National Institutes of Health; The Cancer Atlas Genome Project (TCGA), (CSER), and the Genomic Medicine Working Group

Kiara Palmer, BA – Public Affairs Specialist & Video Producer at National Institutes of Health's (NIH); at the National Human Genome Research Institute.

Tejinder Rakhra-Burris, MA - is the Program Leader of the Duke Center for Personalized and Precision Medicine (CPPM) with the Center for Applied Genomic & Precision Medicine; at Duke University and has over 10 years of experience in administration and program management of scientific research in higher education. In addition to participating in the strategic and administrative /operational leadership of the CPPM, she also is the program manager for the NHGRI-funded genomic demonstration project entitled "Implementation, Adoption, and Utility of Family History in Diverse Settings" and oversees the management of two United States Air Force cooperative agreements. Prior to joining Duke, she was the Associate Director for Research with the University of North Carolina Institute for Pharmacogenomics and Individualized Therapy, and previously managed a CDC-funded public health genetics program at the UNC School of Public Health, and an NIEHS-funded cooperative agreement into the feasibility of establishing a national twin registry for genetic and environmental association studies. Ms. Rakhra-Burris has a background in business administration and a Master's Degree in Molecular Genetics from Washington University in St. Louis.

Alvaro Encinas - the Visual Professional at the National Human Genome Research Institute.