An updated SYSCILIA gold standard (SCGSv2) of known ciliary genes, revealing the vast progress that has been made in the cilia research field

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ABSTRACT **Cilia are microtubule-based organelles with important functions in motility and sensation. They contribute to a broad spectrum of developmental disorders called ciliopathies and have recently been linked to common conditions such as cancers and congenital heart disease. There has been increasing interest in the biology of cilia and their contribution to disease over the past two decades. In 2013 we published a "Gold Standard" list of genes confirmed to be associated with cilia. This was published as part of the SYSCILIA consortium for systems biology study dissecting the contribution of cilia to human health and disease, and was named the Syscilia Gold Standard (SCGS). Since this publication, interest in cilia and understanding of their functions have continued to grow, and we now present an updated SCGS version 2. This includes an additional 383 genes, more than doubling the size of SCGSv1. We use this dataset to conduct a review of advances in understanding of cilia biology 2013– 2021 and offer perspectives on the future of cilia research. We hope that this continues to be a useful resource for the cilia community.**

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INTRODUCTION

Cilia are microtubule-based cell surface organelles with important functions in motility and sensation. There are three subclasses of cilia, defined by their microtubule ultrastructure. First, motile cilia are found in large numbers on the epithelial cells of the reproductive tracts, brain ventricles, and respiratory tract. Cells with motile cilia are often called multiciliated cells (MCCs). These cilia have backbones (axonemes) of rings of nine microtubule doublets, with a central pair of microtubules, and dynein arms allowing the cilia to beat in a coordinated motion to facilitate fluid flow over the cell surface (Legendre *et al.*, 2021). Second, nodal cilia are a population of cilia that exist transiently in embryonic development at the embryonic node. They lack the central pair of microtubules but retain motility and function in directional fluid flow at the node to establish left–right asymmetry in the embryo (Nonaka *et al.*, 1998). Finally, primary cilia are single nonmotile organelles found on the surfaces of all other epithelial cells in the body and some other cell types such as fibroblasts. They lack the central pair of microtubules and dynein arms. They do not beat and their primary functions are in chemosensation, mechanosensation, and, in the retina, photosensation (Wheway *et al.*, 2018). The outer segment of the photoreceptor cell of the retina is a huge and highly specialized primary cilium (Bujakowska *et al.*, 2017).

Until several decades ago, primary cilia were believed to be vestigial organelles with no significant function. They were assumed to have lost their motility and been rendered redundant. This view was challenged in the early 2000s when it was demonstrated that primary cilia are required for normal kidney function, with the discovery that IFT88, mutated in a mouse model of polycystic kidney disease, is required for cilium assembly (Pazour *et al.*, 2000). This led to molecular investigations that identified the primary cilium as a sensory organelle, with mechanosensory roles in the kidney mediated by polycystins in the cilium membrane (Yoder *et al.*, 2002; Nauli *et al.*, 2003). This was an important discovery, as it uncovered the role of the primary cilium in autosomal dominant polycystic kidney disease, one of the most common human genetic diseases (Ong and Wheatley, 2003). Primary cilia have subsequently been shown to be central signaling organelles, with roles in signal transduction

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Abbreviations used: BP, biological processes; GO, gene ontology; IFT, intraflagellar transport; MCC, multiciliated cell; SCGS, SYSCILIA gold standard; v1, version 1; v2, version 2.

in the Hedgehog (Huangfu *et al.*, 2003) and Wnt and PDGFRα signaling pathways (Schneider *et al.*, 2005; Simons *et al.*, 2005). They play important roles throughout development, from very early embryogenesis. The clinical consequences of the aberrant development or function of primary cilia extend beyond polycystic kidney disease to encompass a spectrum of severe inherited human disorders known collectively as the ciliopathies (Oud *et al.*, 2017).

Much of our understanding of the basic structure and function of cilia has derived from the study of ciliated model organisms including the single-celled eukaryotes *Chlamydomonas reinhardtii*, *Paramecium tetraurelia*, *Tetrahymena thermophila*, and *Trypanosoma brucei* (Vincensini *et al.*, 2011). Study of these organisms provided us with the first proteomes of the cilium (Pazour *et al.*, 2005; Smith *et al.*, 2005; Broadhead *et al.*, 2006; Arnaiz *et al.*, 2009) and basal body (Kilburn *et al.*, 2007), constructed using approaches such as high-throughput proteomics and comparative genomics. *Caenorhabditis elegans* and *Drosophila melanogaster* were useful models for early characterization of genes encoding ciliary proteins through genome-wide identification of promoters containing an X-box and study of genes under control of ciliary transcription factors (Blacque *et al.*, 2005; Efimenko *et al.*, 2005; Laurençon *et al.*, 2007). These simple model organisms show some striking conservation with humans and have been useful for identifying and characterizing orthologues of human ciliopathy genes (Keller *et al.*, 2005; Chen *et al.*, 2006), further developed through studies of vertebrate models such as *Xenopus tropicalis*, *X. laevis*, and zebrafish (Song *et al.*, 2016; Rao and Kulkarni, 2021). Rat and mouse have been important mammalian models for understanding and modelling the role of cilia in human health and disease (Norris and Grimes, 2012). Collectively, many high-throughput genomic, proteomic, and gene expression studies in model organisms and humans have contributed to ciliary databases such as Cildb (Arnaiz *et al.*, 2009, 2014) and the ciliary proteome (Gherman *et al.*, 2006) and ciliome (Inglis *et al.*, 2006). These databases comprise lists of genes and proteins identified in high-throughput ciliary studies in different ciliated model organisms and humans, but are generally assembled using computational methods and not curated by human experts (with perhaps the exception of Nogales-Cadenas *et al.*, 2009).

To address this, in 2013 we published version 1 of the SYSCILIA gold standard (SCGSv1; van Dam *et al.*, 2013), a manually curated list of known ciliary components compiled with expert review of each entry. This was born out of a need for a robust positive control set of high-confidence ciliary genes to aid interpretation of the multiple large datasets being produced by hypothesis-neutral screening approaches implemented in the collaborative European research program SYSCILIA (http://www.syscilia.org/index.shtml). This positive control set proved instrumental in quantifying the enrichment of known ciliary genes in these screening results, allowing us to evaluate the success of such screening strategies and confidence in our novel findings (Slaats *et al.*, 2015; Wheway *et al.*, 2015; Boldt *et al.*, 2016; Lambacher *et al.*, 2016). This original list was focused on primary cilia genes, with less consideration of motile cilia genes.

In the eight years since this resource was published, the paper has been a useful resource for other groups analyzing screening data (Gupta *et al.*, 2015; Roosing *et al.*, 2015; Shim *et al.*, 2016; Pusapati *et al.*, 2018; Gheiratmand *et al.*, 2019), for evolutionary genetics studies (Nevers *et al.*, 2017; Shulman and Tsou, 2017), and in prioritization of candidate genes from exome and genome sequencing of ciliopathy patients (Shaheen *et al.*, 2016). The field of ciliary biology has advanced rapidly since the publication of SCGSv1, and in response we now provide an updated SCGS, including updated annotation of all genes, achieved through systematic literature searching and candidate gene analysis. The result is the SCGSv2, listing 686 genes, a major increase from SCGSv1, which contained 303 genes. We advance the utility of this dataset by grouping entries into two main categories, first-order and second-order cilia genes, elaborating a concept first put forward by Reiter and Leroux (2017). Reiter and Leroux propose that ciliopathies fall into two categories: first-order ciliopathies, which are diseases caused by aberrations in genes encoding proteins localized to the cilium, and second-order ciliopathies, which arise as a result of defects in genes that encode proteins not localizing to the cilium but having a role in cilium formation or function. Similarly, we annotate genes as first-order if they encode proteins that localize to the cilium or basal body and second-order if they encode proteins that do not localize to the cilium or basal body but otherwise have roles in cilium structure or function. SCGSv1 was focused on primary cilia genes, and in SCGSv2 we expand this to include motile cilia genes also. We review the new entries to give a perspective on recent advances in understanding of cilium structure and function and the role of cilia in development and disease.

RESULTS AND DISCUSSION

Three hundred eighty-three additional gold standard cilia genes were identified, producing the SCGSv2 of 686 genes, a major increase from SCGSv1 which contained 303 genes (Supplemental Table S1). Five hundred thirty-nine of these 686 (78.6%) are first-order cilia genes, and 133 are second-order (19.4%). Fourteen have not had their protein localization reported and so are not designated as first-order or second-order. A retrospective analysis of SCGSv1 shows that 273 of the 303 genes were first-order cilia genes (90.1%) and 25 were second-order (8.3%). This may suggest that since the publication of SCGSv1 an increasing awareness of cilia outside of the cilia community has led to more study of the cilium functions of proteins. Alternatively or additionally, it may suggest that the discovery of first-order cilium genes is becoming saturated, and so a proportional increase in second-order cilia gene discovery has been seen more recently.

Fifty-seven of the 383 new genes (14.9%) were originally qualified as predicted in the SCGSv1 paper. These 57 genes appeared in experimental and bioinformatics screens without in-depth validation of their function or localization and were provided as an appendix to the SCGSv1. One hundred eighty-seven of the new 383 genes (48.8%) were predicted in CiliaCarta (van Dam *et al.*, 2019), demonstrating the validity of this Bayesian-based approach to predicting ciliary function from genomic, proteomic, transcriptomic, and evolutionary data.

Of the novel cilium genes identified, there are clear trends in the biological pathways that these genes are associated with. An enrichment analysis of Gene Ontology terms (Ashburner *et al.*, 2000; Carbon *et al.*, 2009; The Gene Ontology Consortium, 2019) describing biological processes (GO BP terms) in the list of new cilia genes compared with SCGSv1 shows that the new list of genes is particularly enriched for genes involved in cell stress responses, (de)ubiquitination, autophagy, aging, DNA repair, chromatin remodeling, multiple signaling pathways, and regulation of cardiac growth.

In the new list of cilia genes, one of the most enriched types of genes is those with GO BP terms relating to cellular response to external/environmental stimulus (GO:0071496/GO:0104004, fold enrichment 14.75/6.47, $p = 1.20 \times 10^{-13}/2.54 \times 10^{-10}$). There is particular enrichment of genes with roles in response to stress (GO:0006950, 2.46-fold enrichment, $p = 1.74 \times 10^{-10}$), cellular response to nutrient levels/starvation (GO:0031669/GO:0042594, fold enrichment 10.87/10.09, $p = 5.90 \times 10^{-08}/6.66 \times 10^{-07}$), response

to decreased oxygen levels (GO:0036293, 6.21-fold enrichment, $p = 7.73 \times 10^{-06}$), and cellular response to radiation (GO:0071478, 6.21-fold enrichment, $p = 7.73 \times 10^{-06}$), including DNA repair (GO:0006281, 8.54-fold enrichment, $p = 6.37 \times 10^{-05}$). This suggests a recent increase in understanding of the role of cilia in sensing the cell environment and orchestrating the response to cell stress. In many cases of shock or stress, the literature suggests that rapid responses such as ciliogenesis or cilium resorption are executed via rapid protein degradation via the ubiquitin–proteasome system (UPS). For example, it has been shown that MIB1, which represses ciliogenesis by ubiquitinating CEP131 and PCM1 at centriolar satellites, is abruptly inactivated in response to cell stress, leading to loss of CEP131 and PCM1 ubiquitination and stimulation of ciliogenesis, even in proliferating cells (Villumsen *et al.*, 2013).

Indeed, in addition to MIB1 E3 ligase, many more other genes involved in ubiquitination and even more involved in protein deubiquitination (GO:0016579) are found in SCGSv2 than in SCGS1 (10.87-fold enrichment, $p = 5.90 \times 10^{-08}$). MIB1-mediated ubiquitination and degradation of PCM1 during serum starvation–induced ciliogenesis is antagonized by deubiquitinating enzyme USP9X (Wang *et al.*, 2019). USP9X further contributes to cell cycle–dependent ciliogenesis; in the G0/G1/S phase, USP9X is recruited to the centrosome by NPHP5, where it protects NPHP5 from ubiquitination, promoting cilia assembly. In the G2/M phase, USP9X dissociates from the centrosome, allowing BBS11/TRIM32 (E3 ligase) to K63 ubiquitinate NPHP5, triggering protein delocalization and loss of cilia. USP14 has been shown to control ciliogenesis, cilia length, and localization of mediators of Hedgehog (Hh) signaling in cilia through deubiquitination and stabilization of KIF7 (Massa *et al.*, 2019). USP8 deubiquitinates HIF1a to control ciliogenesis in normoxia (Troilo *et al.*, 2014) and antagonizes Smo ubiquitination (Ma *et al.*, 2016). SUMOylation has also been implicated in the trafficking of Smo into cilia (Ma *et al.*, 2016), further broadening our understanding of how protein degradation pathways control cilium structure and function. The increase in understanding of the protein modifications involved in protein metabolism relevant to ciliogenesis and cilium function is reflected in the enrichment of terms in SCGSv2 related to positive regulation of phosphorylation (GO:0042327, 3.98-fold enrichment, $p = 7.59 \times 10^{-11}$), positive regulation of kinase activity (GO:0033674, 4.97-fold enrichment, $p = 1.46 \times 10^{-10}$), protein modification by small protein removal (GO:0070646, 11.64-fold enrichment, $p = 4.87 \times 10^{-09}$), and positive regulation of protein modification process (GO:0031401, 2.98-fold enrichment, $p = 4.33 \times 10^{-08}$).

In the years since the publication of SCGSv1, it has also become apparent that autophagy plays a role in this rapid ciliogenesis/cilium resorption process. Indeed, genes with GO BP terms relating to regulation of autophagy (GO:0010506) are enriched in the new additions to SCGSv2 (3.3-fold enrichment, $p = 1.45 \times 10^{-02}$). This includes ATG3 and ATG5, which are required for rapid degradation of OFD1 at centriolar satellites in response to serum starvation (Tang *et al.*, 2013). This landmark publication in *Nature* led to a suite of papers in recent years describing autophagic processes removing "cilia roadblocks" to promote ciliogenesis, control cilium length, and control cell volume (Jang *et al.*, 2016; Orhon *et al.*, 2016; Hsiao *et al.*, 2018; Liu *et al.*, 2018; Struchtrup *et al.*, 2018; Boukhalfa *et al.*, 2020). The interest in autophagy of cilia components has even led to the suggestion of a specific term for this process, "ciliophagy" (Cloonan *et al.*, 2014). It has long been observed that serum starvation can induce ciliogenesis in cell culture, and this recent work studying the UPS, the SUMOylation pathway, and autophagy has provided insights into the mechanisms and dynamics of this process.

While cilia have been recognized as signaling hubs for a number of years now, the extent to which the cilium plays a role in almost every signaling pathway in the cell was perhaps unprecedented. Since the publication of SCGSv1, the cilium has been reported as playing a role in IGF signaling (Yeh *et al.*, 2013), FGF signaling (Kunova Bosakova *et al.*, 2019),, Hippo/YAP/TAZ signaling (Kim *et al.*, 2015), prostaglandin signaling (Jin *et al.*, 2014), notch signaling (Boskovski *et al.*, 2013), mTOR signaling (Zhong *et al.*, 2016; Park *et al.*, 2018) and TGFbeta signaling (Clement *et al.*, 2013). TGF-beta signaling through the cilium was shown to be important for cardiomyogenesis (Clement *et al.*, 2013), and GO BP terms relating to cardiac muscle growth (GO:0055021, 6.99-fold enrichment, $p = 4.77 \times 10^{-03}$) are also enriched in the new cilia genes of SCGSv2. While one of the earliest discoveries in 9 + 0 cilia biology was the role of nodal cilia in establishing leftward nodal fluid flow, breaking left–right symmetry for proper heart looping (Nonaka *et al.*, 1998), more recently there have been advances in understanding of the role of primary cilia in later heart development and function and the contribution of cilia defects to congenital heart disease (Li *et al.*, 2015; Scott *et al.*, 2017; Toomer *et al.*, 2019). Overall, however, there is significant underrepresentation of genes involved in developmental processes such as brain development, limb morphogenesis, heart looping and left–right asymmetry in the new cilia gene list compared with SCGS1, suggesting that in recent years smaller gains have been made in understanding of the role of cilia in early developmental processes.

Enrichment of genes with the GO BP terms chromatin organization (GO:0006325, 11.64-fold enrichment, $p = 4.87 \times 10^{-09}$), histone modification (GO:0016570), and covalent chromatin modification (GO:0016569; both 9.32-fold enriched, $p = 6.97 \times 10^{-06}$) in SCGSv2 represents an increase in understanding of transcriptional regulation of ciliogenesis, and also of the dual roles of histone-modifying enzymes in histone modification and other functions in the cilium. This includes KDM5C, which is involved in regulating ciliogenesis by regulating actin gene expression, and also through directly binding to the actin cytoskeleton, creating a responsive "actin gate" that involves ARP2/3 activity and IFT (Yeyati *et al.*, 2017), and TRRAP, an essential component of multiple histone acetyltransferase complexes, which regulates multiciliated cell formation (Wang *et al.*, 2018). There has also been an increase in understanding of how various transcription factors regulate ciliogenesis, such as MCM2, which binds to transcription start sites of cilia-inhibiting genes to control ciliogenesis in postmitotic cells (Casar Tena *et al.*, 2019), and MYB transcription factor, which plays a role in multiciliogenesis, as progenitors exit the cell cycle and amplify their centrioles (Tan *et al.*, 2013). Furthermore, transcription factor ATOH1 controls ciliogenesis in neuron progenitors (Chang *et al.*, 2019) and transcription factor SREBF1 activates expression of PLA2G3 to repress cilium formation in cancer cells (Gijs *et al.*, 2015). Recent studies have also expanded understanding of the role of RFX transcription factors RFX2 and 7 in regulating coordinated ciliogenesis (Chung *et al.*, 2014; Manojlovic *et al.*, 2014). Furthermore, it has been shown that some transcription factors have secondary functions in cilia, such as SALL1 transcription factor, which interacts with factors related to cilia function, including the negative regulators of ciliogenesis CCP110 and CEP97 (Bozal-Basterra *et al.*, 2018). Additionally, post-trancriptional regulation of cilia genes is beginning to be understood with the discovery that pre-mRNA splicing factors regulate splicing of cilia genes (Wheway *et al.*, 2015; Buskin *et al.*, 2018) and NUDT16L1 (SDOS) posttranscriptionally regulates cilia genes by binding and regulating translation of cilia mRNAs (Avolio *et al.*, 2018).

Finally, it is an interesting observation that the new genes in SCGSv2 are enriched for the GO BP term aging (GO:0007568, 9.32-fold enrichment, $p = 6.97 \times 10^{-06}$). While there are few publications directly linking cilia to aging (Carroll and Korolchuk, 2018), it is well known that the cilium plays a central role in nutrient sensing, and reduced responsiveness of nutrient-sensing pathways is associated with aging. The nutrient-sensing role of cilia in aging may become more apparent in future research. Furthermore, recent research has linked cilia defects to induction of cell senescence (Jeffries *et al.*, 2019) and conversely has shown that depolarization of senescent cell plasma membrane leads to primary cilia defects and a resultant failure to inhibit growth factor signaling (Carroll *et al.*, 2017). Senescence has been described as a feature of some ciliopathies such as nephronophthsis type 7 (Lu *et al.*, 2016). This is significant, because cilia are classically associated with developmental disorders, yet may also play a role in aging and associated disease, which are some of the most costly burdens to our society today, both economically and socially.

The aim of this study was to produce a high-confidence list of cilia genes annotated by cilia experts. The approach prioritized the exclusion of false positives over the exclusion of false negatives, and as a result the list is highly stringent and not a completely comprehensive list of all cilia and basal body genes. Absence of a gene from this list does not necessarily mean that gene does not play a role in ciliogenesis or cilium structure or function, but inclusion of a gene in this list means that it is highly confidently associated with these processes in humans. The literature search did not include gray literature or literature in preprint servers before peer review, and as a result, the most recently identified and characterized genes will not be included. The literature search focused on human genes (with "human" included as search term) and the titles of search results were reviewed for mention of genes in humans or vertebrate models so that cilia genes that have been described in model organisms but for which the orthologue has not been well characterized in humans/human cell lines will be omitted. The resulting list is a stringent, high-confidence list of genes involved in ciliogenesis and cilium structure and function with a focus on human cilia and ciliopathy genes. For more comprehensive lists of genes that include candidate genes, likely false positives, and genes that have no orthologue in humans, we direct the reader to CiliaCarta (van Dam *et al.*, 2019) or cildb (Arnaiz *et al.*, 2014).

MATERIALS AND METHODS

Request a protocol through *Bio-protocol*.

On 1 January 2021, a systematic review of Medline was conducted using the following MESH terms: ((((((((((((((((((cili*[Title/Abstract]) NOT ciliary body[Title/Abstract]) AND ("2013/05/01"[Date-Publication]: "3000"[Date-Publication])) AND English[Language]) AND Humans[Mesh])) NOT ciliate[Title/Abstract]) AND Humans[Mesh])) NOT ciliary muscle[Title/Abstract]) AND Humans[Mesh])) NOT Ciliary Neurotrophic Factor Receptor[Title/Abstract]) AND Humans[Mesh])) NOT cilioretinal[Title/Abstract]) AND Humans[Mesh])) NOT ciliochoroidal[Title/Abstract]. This returned 4548 results. Each title was assessed for mention of gene names, or for the word "screen." Where novel genes or screen results were reported in vertebrates, human cells, or human cell lines, abstracts and figures were studied to identify the nature of protein function and immunofluorescence or immunogold electron microscopy images showing the localization of the protein. Official gene symbol, Ensembl gene ID, any associated OMIM ID, curators note, relevant PubMed IDs, and localization were recorded in a spreadsheet.

In addition to the systematic literature search, the 286 genes predicted to be cilia genes in the CiliaCarta (van Dam *et al.*, 2019) study were specifically included in a Medline search using the MESH terms cili*[Title/Abstract]) AND [gene name 1] OR [gene name 2] … OR [gene name *n*]. Every paper from this search was studied in depth to identify any reported protein functions in cilia and localization.

Once all genes were extracted from this systematic review into a results table (Table S1 in the Supplemental Material), every entry was independently reviewed by a second cilia expert, who entered additional data and annotated the "curators note" column on this table.

If the protein localization was reported as cilium, axoneme, basal body, or part thereof, the gene was scored as a first-order cilium gene. If a protein's localization was reported as any other cell location, including centriole, centrosome, and centriolar satellite, but not explicitly basal body, the gene was scored as a second-order cilium gene.

Once finally compiled, Ensembl gene IDs were filtered to identify which we predicted in the SCGSv1 paper, and which were predicted in the CiliaCarta paper.

Gene Ontology enrichment analysis of SCGSv2 was conducted using amiGO (Carbon *et al.*, 2009) Enrichment of gene ontology terms relating to biological processes in SCGSv2 compared with SCGSv1 was conducted using a binomial test, with Bonferroni correction of the p value to account for multiple testing. Ensembl gene IDs were used as input in amiGO.com which accesses the Panther database.

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