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Bending, David; Zikherman, Julie

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Review

Nr4a nuclear receptors: markers and modulators of antigen receptor signaling

David Bending[1] and Julie Zikherman[2]

Nr4a1–3 encode a small family of orphan nuclear hormone receptors with transcriptional activity. Their expression reflects both acute and chronic antigen-receptor signaling in T and B-cells, and they have been implicated in critical aspects of lymphocyte development, tolerance, and function. These include roles in regulatory T-cell (Treg), thymic-negative selection, humoral responses, anergy, and exhaustion. Here, we review recent advances in this field such as functional roles in B-cells, transcriptional targets, and mechanism of action. We highlight recurrent themes, including integration of antigen-receptor signaling with costimulatory input, as well as unanswered questions and translational applications of this work.

Addresses
[1] Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK
[2] Division of Rheumatology, Rosalind Russell and Ephraim P. Engleman Arthritis Research Center, Department of Medicine, UCSF, San Francisco, CA 94143, USA

Corresponding authors: Bending, David (d.a.bending@bham.ac.uk), Zikherman, Julie (julie.zikherman@ucsf.edu)

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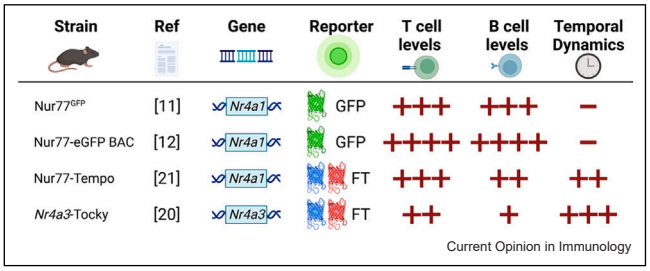
Introduction

The nuclear receptor subfamily 4A (Nr4a) receptor family members Nur77, Nurr1, and Nor1 (respectively encoded by *Nr4a1–3*) are a unique group of nuclear receptors that are rapidly upregulated in T and B-cells following antigen-receptor signaling. They are orphan nuclear receptors with a highly conserved DNA-binding motif but are not thought to rely upon endogenous ligands for their activity [1]. Structural studies suggest that the ligand-binding pocket of Nr4a receptors is physically obscured [1,2]. While originally linked to the induction of apoptosis in T-cell hybridomas [3,4], Nr4a receptors have been shown to play many critical roles in lymphocyte development, function, metabolism, and in promoting immunological tolerance. In this review, we will emphasize recent advances within the field (see reviews [5–7] for greater historical depth), focusing primarily on Nr4a receptor function in B and T lymphocytes. We aim to identify common themes across their biology and highlight questions that remain unanswered. We focus on their functional redundancy in T and B-cells and emphasize their role in restraining lymphocytes that receive antigen-receptor signaling in the absence of costimulation. This review will position Nr4a receptors as key modulators of Nuclear factor of activated T-cells (NFAT) and Activator protein 1 (AP-1)- driven transcriptional networks, making them promising candidates for therapeutic targeting.

Regulation of expression

Nr4a receptors lack well-defined endogenous ligands and — as primary response genes — exhibit a dynamic expression pattern in response to mitogenic stimulation that is unique among nuclear receptors. Therefore, it is thought that their function is heavily dependent on their transcriptional regulation. Among the three family members, *Nr4a1* transcript is consistently more abundant in B and T-cells compared with *Nr4a2* and *Nr4a3*, but all three family members are rapidly induced in response to B-cell receptor (BCR) and T-cell receptor (TCR) stimulation [8–10]. Indeed, their dynamic expression has been exploited to generate informative reporters of antigen-receptor signaling. The development of Nur77/*Nr4a1*-green fluorescent protein (GFP) Bacterial artifical chromosome (BAC) Tg reporter mice has facilitated the study of lymphocyte signaling in vitro and in vivo [11,12]. These tools revealed that Nur77-GFP levels can be used as a proxy for antigen-receptor signal intensity, linking signal strength to T- and B-cell developmental checkpoints and activation thresholds [13–19]. While benefiting from the high transcript abundance of *Nr4a1*, Nur77-GFP reporters (due to the long half-life of GFP protein) detect cumulative antigen stimulation, but cannot distinguish recent or active signaling from prior receptor engagement. Recent development of fluorescent timer protein (FT) reporters,

Figure 1



Comparison of Nr4a reporter mice. Expression levels are a reflection of the published reporter intensity levels in the respective T- and B-cell subsets. (Figure created in BioRender.com).

whose short-lived blue fluorescent form can track TCR signal changes occurring over 4–7 h [20, (e.g. Nur77-Tempo [21] and *Nr4a3*-Tocky [20]), has revealed rapid changes in T-cell activation thresholds following stimulation [22]. Comparison of Nur77-GFP and Nur77-Tempo reporters with *Nr4a3*-Tocky mice confirmed the higher relative expression of Nur77 during T-cell development, which may reflect a different (and higher) threshold for activation of *Nr4a3* compared with *Nr4a1* (Figure 1) [23].

NFAT1 Chromatin immunoprecipitation followed by sequencing (ChIP-seq) experiments revealed that the regulatory region of all three *Nr4a* genes can bind NFAT1 [23,24]. Based on analysis of T-cells expressing constitutively active NFAT complexes in T-cells [24] and NFAT pathway inhibitor studies [23,25], the NFAT pathway is necessary and sufficient for *Nr4a2* and *Nr4a3* but largely redundant for *Nr4a1* transcription. NFAT binding to AP-1 is not required for induction of *Nr4a3* in CD4+ and CD8+ T-cells [24], suggesting that chronic antigen stimulation may lead to enhanced *Nr4a2/3* expression. In common, however is that acute induction of all *Nr4a* gene transcripts is attenuated by inhibition of the Extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (Jnk), and Protein Kinase C (PKC) pathways [12,21]. The dependency of *Nr4a3*-Tocky reporter on the NFAT pathway has been exploited to use expression of *Nr4a3* as a switch-like readout for agonist-driven T-cell activation [22], while Nur77 may be suited to study not only T-cell but also B-cells undergoing both tonic and activating signaling [12]. Beyond the scope of this review, signal-dependent post-translational modifications of the Nr4a family members can regulate protein stability, function, and localization with implications for lymphocyte biology.

Expression and function in T-cells

In this section, we discuss the biology of Nr4a receptors in thymic and peripheral T-cell function. Given the profound defects observed in regulatory T-cell development (Treg) in double and triple Nr4a knock out (KO) mice, the functions of Nr4a receptors in Treg must be considered first before their functions on other T-cells can be elucidated.

Role of Nr4as in regulatory T-cells

Perhaps, the clearest illustration of functional redundancy among the Nr4a family members is evident in their impact on Tregs. Both germline and T-cell-specific deletion of *Nr4a1* and *Nr4a3* (+/- *Nr4a2*), but not individual family members, results in near-complete loss of Treg and a severe scurfy-like inflammatory disease in mice [10,26]. Nr4as link agonist selection to Treg fate in the thymus in part by reinforcing *Foxp3* expression in 'labile' precursors and promoting expression of other Treg-defining transcripts [27]. The Nr4as are required not only for Treg generation, but also for Treg maintenance; conditional deletion of Nr4a family members with Foxp3-cre results in loss of *Foxp3* expression and production of inflammatory cytokines [28]. Finally, Nr4as promote peripheral Treg induction while repressing Th1 and Th2 fate by engaging similar transcriptional targets in naive CD4 T-cells differentiated under polarizing conditions [29]. Both Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) and CHIP-seq approaches argue that at least some of these effects are mediated by direct transcriptional regulation of *Foxp3* expression by Nr4a nuclear receptors, although which family members mediate these effects may be context-specific. For example, Treg differentiation in the setting of calcineurin inhibitors requires Nur77/Nr4a1 because of exclusive NFAT dependence of *Nr4a2* and *Nr4a3* [25]. Nur77/Nr4a1 (but not Nr4a2 or 3) has been proposed to mediate Treg induction by a bacterial bile acid metabolite, isoalloLCA, in a manner that requires the CNS3 enhancer of *Foxp3* [30]. Whether isoalloLCA may directly interact with Nr4a1 as an agonist ligand of this 'orphan' receptor remains to be determined. In summary, Nr4a receptors play essential but individually redundant functions in Treg development and function.

Role of Nr4a receptors during T-cell development in the thymus

Nr4as are upregulated by TCR signaling in thymocytes at the positive selection checkpoint, and both endogenous *Nr4a* and reporter expression are highly increased among thymocytes destined for negative selection [11,31–33]. Despite differences in transcript abundance, studies reveal remarkable functional redundancy between Nr4a1 and Nr4a3 receptors during negative selection. By contrast, Nurr1/Nr4a2 protein is reported to be undetectable in stimulated thymocytes [31]. Indeed, one of the earliest roles identified for the Nr4a family was as mediators of antigen-induced cell death in T-cells; overexpression of full-length Nr4a family members triggered apoptosis in both T-cell

hybridomas and thymocytes, while a dominant-negative construct of Nur77 harboring an n-terminal truncation but retaining the DNA-binding domain blocked antigen-induced cell death in both settings [3,31,34,35]. Strikingly, when *Nr4a1*-/- mice were first characterized, no defect in thymic deletion in the H-y antigen (H-Y) and AND TCR Tg models could be identified, suggesting profound redundancy among the family members. Subsequent studies did identify modest defects in negative selection of *Nr4a1*-/- (s)KO and *Nr4a3*-/- (s)KO thymocytes in response to membrane-bound form of OVA under the control of the rat insulin promoter (RIPmOVA) transgene [36,37], suggesting that reduced stringency of deletion and/or mTEC-dependent deletion pathways were more dependent upon the Nr4a family. Nevertheless, discrepancy between phenotypes of (s)KO models and dominant-negative Nur77 constructs remained. The study of thymic selection in mice lacking multiple Nr4a members was hampered by severe Treg deficiency, systemic inflammation, and associated thymic atrophy. Recently, a competitive chimera strategy was deployed to reconstitute Treg of WT origin to unmask the role of Nr4a1/3 redundancy during thymic selection [10]. This revealed a profound defect in negative selection of polyclonal (d)KO thymocytes at the SP stage in response to endogenous antigen.

Although Nr4as and BIM/*Bcl2l11* are among the few confirmed 'executioners' of self-reactive thymocytes, the mechanism by which Nr4as mediate negative selection and how they link to BIM remains an important open question. Early on, Nr4a-mediated deletion was found to be Fas-independent [31,35]. Dominant-negative Nur77 constructs inhibit negative selection to stringent ubiquitous model antigens; because these constructs retain DNA-binding domain and block the transcriptional activities of other Nr4a family members, it was presumed that the mechanism by which they evade deletion is transcriptional [31,34,35]. However, the transcriptional target(s) that mediate this phenotype remain unclear [38]. *Bcl2l11* expression is reduced in negatively selecting *Nr4a1*-/- (s)KO thymocytes but only twofold, leaving open the question of whether Nr4as function upstream of BIM [37]. Moreover, genetic epistasis studies of *Nr4a1* and *Bcl2l11* have not resolved this conundrum [37,39,40]. In parallel, Nr4as were shown to translocate to mitochondria and induce a conformational change in Bcl-2 through direct interaction, exposing its BH3-only domain and triggering apoptosis in a non-transcriptional manner [41,42]. Circumstantial, but indirect, data link this mechanism to thymic-negative selection [43]. Transcriptional targets that definitively mediate Nr4a-dependent cell death in the thymus might yet be revealed by deletion of both *Nr4a1* and *Nr4a3* to overcome functional redundancy. How this mechanism is influenced by stringency of the deleting signal as well as context and stage at which the antigen is presented (e.g. medullary thymic epithelial cell (mTEC), other antigen presenting cells (APCs)) remains to be determined.

Nr4a receptor function in peripheral T-cells

Because Nr4as have redundant functions during thymic selection, defining their roles in peripheral T-cells has been challenging. Nr4a1-sKO mice have revealed that Nur77 can act as a brake on the remodeling of metabolic pathways during T-cell activation [44], a result consistent with a role for Nur77 in regulating enzymes involved in glycolysis [37]. Nr4a3-sKO mice display alterations in early CD8+ T-cell differentiation [45]. Much stronger phenotypic differences have been observed with double (d)KO and triple (t)KO T-cells, but require complex conditional and chimera strategies to overcome developmental impacts on Treg and thymic selection. Analysis of competitive chimeras containing both wild type and Nr4a1/3 (d)KO bone marrow revealed WT-derived Treg compartment with highly self-reactive (d)KO peripheral T-cells that escaped negative selection and instead acquired phenotypic and transcriptional hallmarks of anergy — albeit with an increased inflammatory gene signature and a heightened propensity to make IL-2 upon stimulation [10]. A common theme across all KO studies is the suppression of early effector cytokines such as IL-2 [8,10] and IFN-γ [8,44,45], indicating an immediate early negative feedback role for Nr4a receptors on the T- cell activation process.

Nr4a family receptors have also been linked to chronically Ag-stimulated T-cells. Nur77 expression marks self-reactive arthritogenic T-cells in mice and humans [15]. Nr4a3 is also persistently expressed in MOG-reactive T-cells within the conserved non-coding sequence (CNS) of mice during EAE onset [20]. Using Nur77-GFP levels as a proxy for relative basal TCR signaling in CD4+ T-cells in vivo reveals that the most strongly signaled CD4+ T-cells produce less IL-2 and express PD1 and anergy-associated markers such as Cbl-b [46]. In addition, chronic stimulation of CD8+ T-cells or strong tolerogenic stimulation of CD4+ T-cells has also been linked to the development of reduced TCR signal responsiveness in vivo (as evidenced by reduced Nur77 or Nr4a3 expression), which can be partially restored by the blockade of coinhibitory receptors such as PD1 or PD-L1 [22,47].

Not only do *Nr4a* transcripts, proteins, and reporters mark chronically stimulated T-cells, but endogenous Nr4as contribute to functional hyporesponsiveness associated with this state. The most highly signaled naive CD4+ T-cells exhibit an accessible chromatin pattern enriched for Nr4a-binding sites [48]. In response to chronic antigen stimulation, Nr4a1 contributes to T-cell 'dysfunction' — promoting expression of both anergy

and exhaustion-associated genetic programs — and may do so by localizing at AP-1 transcription factor-binding sites and repressing AP-1-induced effector genes, thereby enhancing NFAT-only directed tolerogenic gene expression [8]. A similar mechanism was invoked to account for the impact of Nr4as on Th differentiation [29]. Conversely, deletion of all three Nr4a receptors in chimeric antigen receptor T-cell (CAR T) cells evades the NFAT-induced exhaustion program, facilitating tumor clearance, and is associated with enrichment for accessible bZIP (AP-1) and Nuclear factor kappa B (NF-kB)-binding sites rather than NFAT and Nr4a sites that are normally more accessible in exhausted cells [49]. This role entails a feedforward loop between Nr4as and Thymocyte selection-associated high mobility group box (TOX) transcription factor (TF)s [50]. More recently, deletion of Nr4a3 together with Blimp1/Prdm1 was shown to promote Tcf7-associated 'stemness' and limit terminal exhaustion of CAR T [51]. These findings suggest that an important function of Nr4a receptors in peripheral T-cells may be to abrogate AP-1 activity — directly or indirectly — such that high and sustained Nr4a receptor expression augments the NFAT exhaustion program in cooperation with, and redundantly with a network of other TFs [24].

Expression and function in B-cells

As in T-cells, acute antigen-receptor stimulation induces Nr4a family expression in B-cells, evident via endogenous transcript, protein, and reporter expression [9,12,52]. Among the family members, Nr4a1 is again most abundant at rest and after stimulation, followed by Nr4a3, while Nr4a2 expression is extremely low [9]. Chronic Ag stimulation of both naturally occurring and BCR Tg self-reactive B-cells is marked by Nur77-eGFP reporter expression [12,19]. Unlike T-cells, B-cells are responsive to many mitogenic stimuli apart from antigen, including pathogen-associated molecular patterns (PAMPs) such as LPS and these stimuli also induce Nr4a expression in B-cells [12,53]. Nr4a1 and Nr4a3 play additive roles restraining survival and proliferation of B-cells that acutely receive signal 1 (Ag) in the absence of signal 2 (costimulation via T-cell help or PAMPs) [9]. Similarly, Nr4as expressed by self-reactive B-cells in response to chronic signal 1 reduce survival when the supply of the B-cell survival factor B-cell activating factor (BAFF) is limiting [19]. Conversely, provision of abundant signal 2 bypasses this restraining mechanism. *Batf* and *Myc* are among key transcriptional targets of Nr4as that mediate this negative feedback loop downstream of BCR stimulation [9]. Surprisingly, Nr4as also dampen BCR-induced upregulation of CD86, ICAM1, and the T-cell chemokines CCL3 and CCL4, and this is associated with an advantage for Nr4a-deficient B-cells in competition for a limiting supply of T-cell help [9]. Indeed, Nr4as function to restrain immunodominant B-cell clones at early timepoints in a polyclonal immune response, and thereby promote participation of lower-affinity/avidity subdominant clones [54]. This may be an important mechanism to preserve lower-affinity but potentially neutralizing antibody responses elicited by infection. Such a negative feedback loop operating in B-cells may also preserve clonal diversity in the germinal center reaction, which would be predicted to optimize affinity maturation in the long run. Indeed, BCR-dependent Nur77-eGFP reporter expression is also evident among light zone (LZ) Germinal centre (GC) B-cells encountering follicular Dendritic Cell (FDC), antigen, and Tfh [13,54]. It remains to be formally determined what role the Nr4a family may play during the GC response, but a role in mediating Ag-induced cell death could serve to delete self-reactive clones that arise de novo or have been recruited into the GC. Regulation of targets such as Batf, Myc, and CD86 by Nr4as in acutely activated B-cells might predict functional significance for the Nr4as in LZ GC B-cells.

Discussion

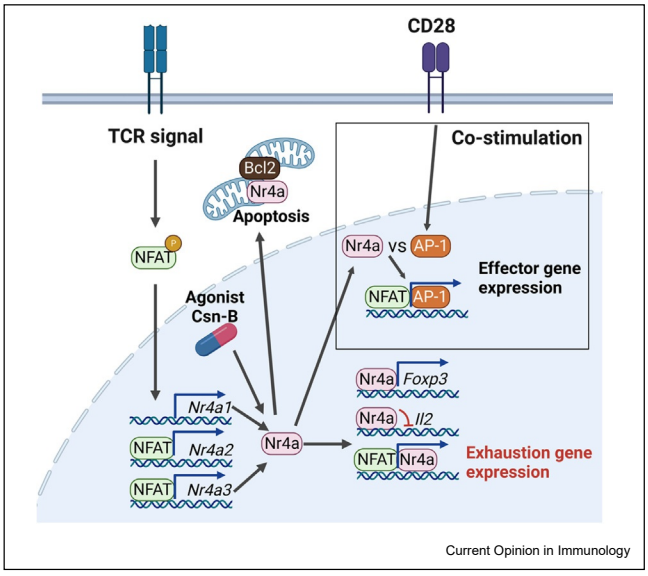
Common themes

Across developmental stages and spanning both acute and chronic antigen stimulation, the Nr4as play coherent, tolerogenic roles during thymic-negative selection, in Treg, in peripheral T- and B-cell anergy/exhaustion, and following acute antigen stimulation of naive lymphocytes. A common theme is that Nr4as restrain lymphocytes that receive signal 1 in the absence of signal 2 — a well-appreciated trigger for a gene expression program that imposes anergy/unresponsiveness mediated by NFAT signaling in the absence of AP-1 (Figure 2). Recent work has begun to uncover a transcriptional 'logic' that incorporates Nr4as as modulators of this network downstream of NFAT. Indeed, Nr4a targets across multiple contexts include induction of checkpoint molecules and suppression of effector cytokines.

Unanswered questions

Nevertheless, many questions about Nr4a function in lymphocytes remain to be addressed. Although *Nr4a1* transcript is much more abundant than *Nr4a3*, these two family members exhibit substantial redundancy in Treg and during thymic selection. By contrast, the Nr4as display more additive effects in peripheral T and B-cells. Why do family members compensate for one another in certain settings but not others? What are the unique characteristics of individual Nr4a proteins and their targets that may account for these discrepancies? Another unexplored function of Nr4as relates to their role downstream of mitogenic receptors other than antigen receptors, and particularly in innate-like lymphocytes that rely more heavily on such pathways. For example, the role of Nr4as downstream of TLRs in B-cells has yet

Figure 2



Regulation and function of Nr4a receptors in T-cells. T-cell receptor signaling leads to the nuclear translocation of NFAT, which is required for the activation of *Nr4a2* and *Nr4a3* but is redundant for *Nr4a1* gene expression. Nr4a receptors can under certain conditions bind and directly promote *Foxp3* expression. Nr4a receptors directly repress *Il2* and *Ifng* transcription and in situations of chronic antigen stimulation can co-opt the NFAT-induced gene exhaustion program. We propose that costimulation enhances AP-1 activity that will compete with Nr4a receptors for access to AP-1-binding sites and therefore promote T-cell effector gene expression. Nr4a receptors can also interact with Bcl-2, causing a change in its BH3 domain and triggering apoptosis. Csn-B is a chemical agonist that could be used to increase Nr4a receptor function. (Figure created in BioRender.com).

to be uncovered, and functions downstream of immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors in NK cells and innate lymphoid cells (ILCs) are unknown.

Agonist-selected lymphocyte populations such as Treg express high levels of Nr4as and depend on them, but other agonist-selected T-cell populations such as NKT express high levels of Nr4as, yet the function of the Nr4as is not fully explored in these cell populations [11,55,56]. Moreover, since Nr4as mediate both agonist-dependent deletion of self-reactive thymocytes and their diversion to the Treg lineage, how are these nuclear receptors directed toward one or the other of these roles with presumably distinct transcriptional targets and/or cytosolic mechanisms? B1a cells are an innate-like B-cell population with a fetal origin that are selected for self-reactivity with high expression of Nur77-eGFP reporter [14]. In B1a cells, Nur77 restrains differentiation into IgM plasma cells, but the transcriptional mechanism is unknown as are the roles Nr4as may play in MZ B-cells that also exhibit high Nur77-eGFP expression [12,14,57].

Recently, Nr4a expression in human B-cells has been identified both in Systemic lupus erythematosus (SLE) patients and among ectopic lymphoid structures in RA synovium [58,59]. It will be important to understand whether Nr4as mark self-reactive human lymphocytes, and whether their transcriptional targets and functions mirror those defined in mice. Finally, although common genetic variants in Nr4as are not among well-recognized loci identified in GWAS for immune-mediated diseases, rare genetic variants that impact Nr4a family member genes could play a role in human immune-mediated diseases and will be of interest to identify as larger and more well-characterized human datasets become available [25].

Ligands and translational applications

Although the Nr4as do not appear to depend upon endogenous ligands for their transcriptional activity and regulation, modulators have been reported (recently reviewed in [60]), including putative endogenous ligands such as prostaglandins [61,62], as well as exogenous ligands such as cytosporone B (Csn-B) [63]. Some ligands are proposed to bind at noncanonical sites with a broad range of K_D and to influence protein–protein association rather than transcriptional activity as conventional hormone receptor ligands do. Both agonist and antagonist compounds, particularly those with selective affinity for specific Nr4a family members, might find numerous applications. For instance, brief inhibition of Nr4as could function as adjuvants for T-cell independent (TI) vaccines, while Nr4a agonists could enhance clonal diversity in response to T-cell dependent (TD) immunizations. Similarly, Nur77 agonists could promote T- and B-cell tolerance in the setting of transplant and autoimmunity. Indeed, active efforts are underway to identify new Nur77 ligands with small-molecule screens [64]. As proof-of-principle, Nur77 agonist Csn-B has been studied across a range of animal models of inflammatory disease (reviewed in [65]). Exploiting Nr4as as drug targets is no longer limited to bona fide ligands; with the advent of CAR T-cell engineering and design of ubiquitin ligase-based degraders, Nr4as could be eliminated in specific cell types for cancer immunotherapy to reinvigorate exhausted T-cells. Future work to refine our understanding of Nr4a functions in mouse and human lymphocytes will pave the way toward targeted therapeutic applications.

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Data Availability

No data were used for the research described in the article.

Conflict of interest statement

JZ is a scientific advisor for Walking Fish Therapeutics. DB declares no conflicts of interest.

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