THY1/CD90 SURFACE GLYCOPROTEIN: SENSOR OF THE MICROENVIRONMENT?

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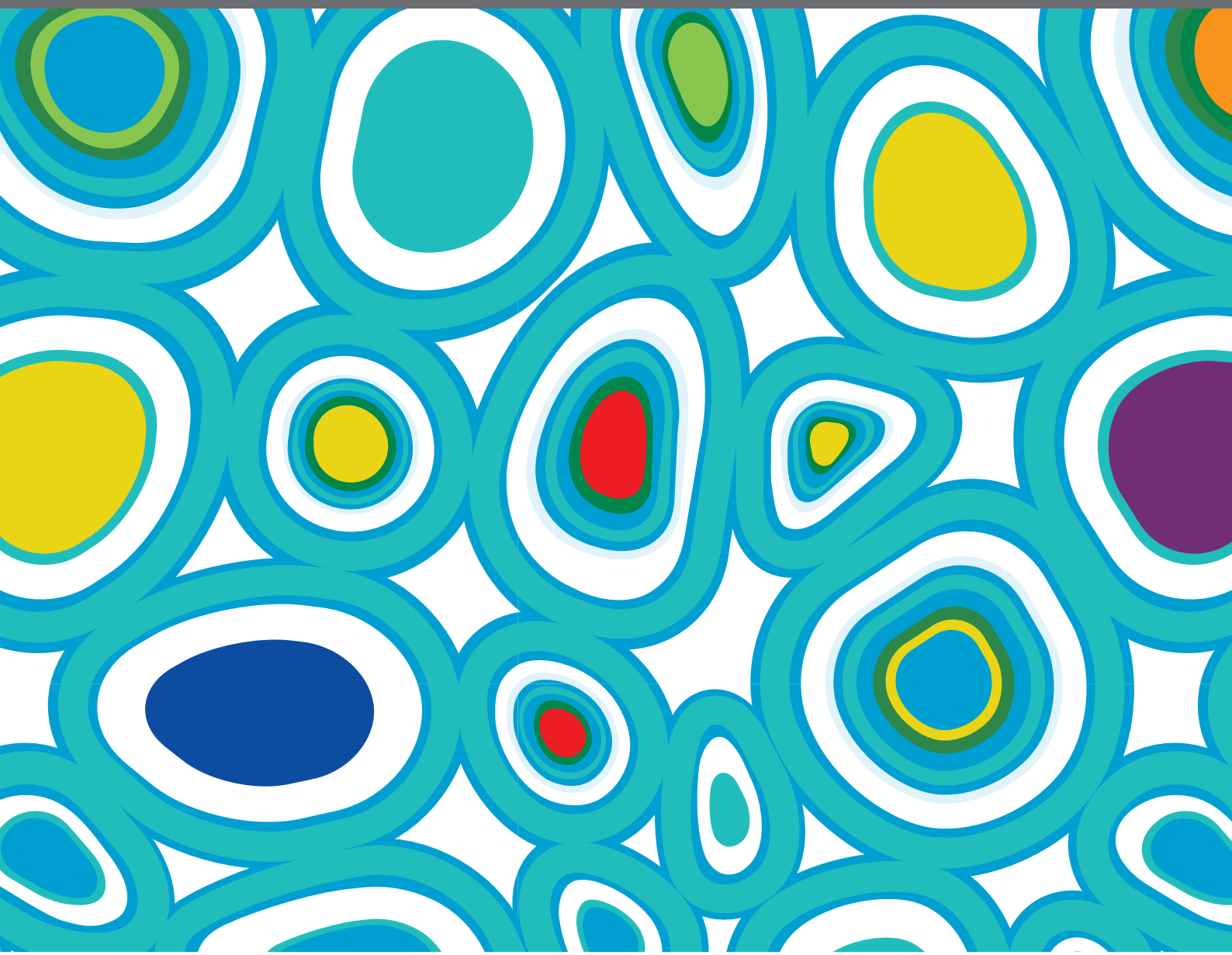

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THY1/CD90 SURFACE GLYCOPROTEIN: SENSOR OF THE MICROENVIRONMENT?

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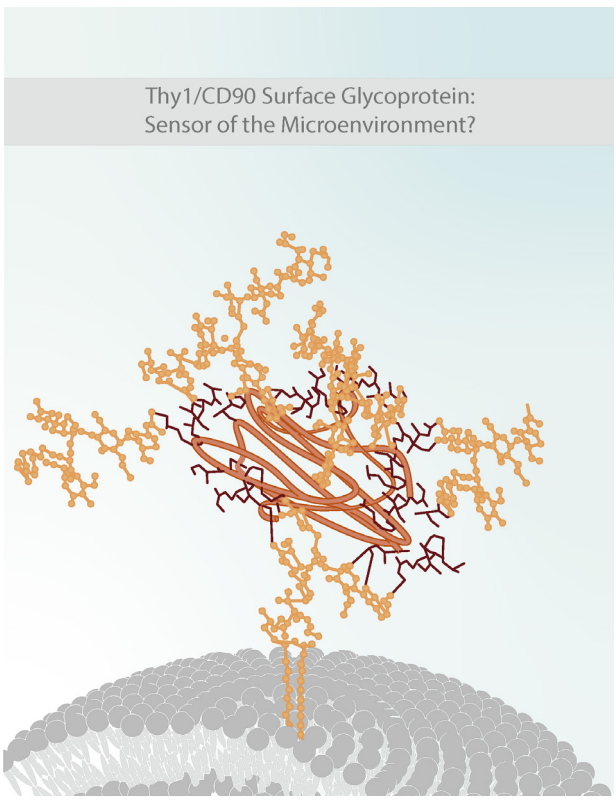

Thy1/CD90 Surface Glycoprotein: Sensor of the Microenvironment?
Image: Bonhomia Design, bonhomiadesign@outlook.com

Thy1/CD90 is a small and heavily N-glycosylated protein that was first identified on the cell surface of murine thymocytes. Today, it is known to be expressed in several other cell types, including human fetal thymocytes, hematopoietic stem cells, some subsets of fibroblasts, liver stem/progenitor, neurons, glioblastoma, mesothelium precursors and mesothelioma cells. In some tissues, Thy-1/CD90 plays a role in different processes, such as cell adhesion, interaction with extracellular matrix, migration. However, despite the available insights to the function of the molecule, we still require a better understanding of the processes that drive Thy1/CD90 expression and the consequences of its presence in cells, as well as to what

extent these functions are conserved between species. The latter is especially relevant when thinking about possible translational studies.

In this Research Topic, various aspects covering available and missing knowledge related to the molecular mechanisms controlling Thy1/CD90 expression and its role in physiological and pathological conditions are discussed.

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EDITORIAL
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Editorial: Thy1/CD90 Surface Glycoprotein: Sensor of Microenvironment?

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Keywords: membrane organizer, membrane micro domains, signaling platforms, cell adhesion molecule (CAM), Thy-1/CD90

Editorial on the Research Topic

Thy1/CD90 Surface Glycoprotein: Sensor of Microenvironment?

Thy-1/CD90 is a small protein modified with lipids and carbohydrates that, as revised by Morris, represents up to 80% in molar terms of the total cell surface protein in thymocytes, from where its name derives. Indeed, a recent single cell transcriptome analysis of 20 mouse organs (The Tabula Muris Consortium et al., 2018) https://tabula-muris.ds.czbiohub.org/, indicates that immature T cells express overall the highest levels of Thy-1/CD90. Despite that, other cells express Thy-1/CD90, and several aspects concerning Thy-1/CD90 molecular interactions, signaling properties, and function in various cell types have been explored/reviewed in this Research Topic. This Frontiers in Cell and Developmental Biology issue includes novel data (Furlong et al; Ilic et al.; Picke et al.), as well as reviews and opinion papers (Jiang and Rinkevich; Hagood; Hu and Barker; Leyton et al.; Morris).

Thy-1/CD90 plays a role in lymphocytes differentiation. Furlong et al. investigate the impact of Thy-1/CD90 vs. T-cell-receptor (TcR) signaling on the production of the T helper (Th) cell subset-associated cytokines, and on the *in vitro* polarization of CD4+ T cells into Th1, Th2, and Th17 cells. The authors proposed the hypothesis that this antigen-independent activation may participate in host-to-host defense against extracellular pathogens. It would be of interest to explore whether this mechanism is involved in modulating immune cells in diseases escaping TcR-mediated T cell activation, such as low mutation tumor burden cancers.

Morris, a pioneer in exploring the function of Thy-1/CD90, calls Thy-1/CD90 "a prime exemplar of a membrane organizer" (Morris) because of its ability to influence and be influenced by its lipid environment. Leyton et al. places it as a "core of a membrane-associated platform (ThyMAP)" based on its capacity to interact with various proteins in the plane of the membrane, with proteins in membranes of opposite cells, and the associations of the multivalent complexes formed, with the extracellular matrix and the cytoskeleton. The regulation of Thy-1/CD90 by its surrounding lipids and proteins leads to fine tuning of the diverse physiological functions of Thy-1/CD90. An example of how the environment can influence Thy-1/CD90 localization is provided by Ilic et al. who, using biochemical assays, offer evidence that modification of a class of glycosphingolipids called gangliosides entails redistribution of Thy-1/CD90 in brain-derived neuronal membranes. In the neurological compartment, Thy-1/CD90 interacts with $\alpha_v\beta_3$ integrin on reactive astrocytes to promote astrocyte adhesion (Leyton et al., 2001; Hermosilla et al., 2008; Lagos-Cabre et al., 2017). An interesting question would be whether this more adhesive phenotype may contribute to manifestation of diseases associated with ganglioside deficiency (Sandhoff et al., 2018).

An intriguing and yet unresolved aspect of Thy-1/CD90 is its expression in mesenchymal stem cells (MSC) and Hagood recalls that the expression of Thy-1/CD90 on MSC has been proposed

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to have several roles. MSC are for example considered precursor cells in bone formation (Chen et al., 2016) and Thy-1/CD90 acts as promoter of bone formation by positively regulating osteoblast differentiation and activation, while concomitantly inhibiting adipogenesis and obesity in mice (Picke et al., 2018). In this Research Topic, Picke et al. provide evidence that under conditions favoring obesity, lack of Thy-1/CD90 impairs bone formation while promoting bone resorption. This obese Thy-1/CD90$^{-/-}$ condition is associated with (i) upregulation of tumor necrosis factor α and (ii) colony stimulating factor 1 (Csf1) expression, which are strong promoters of osteoclast differentiation, and (iii) a reduction in osteoprotegerin (Tnfrsf11b, decoy receptor of RANKL) expression, an inhibitor of osteoclast differentiation. In addition, MSC control the inflammatory response by contributing to the immunosuppressive environment (Estrela et al., 2017). As revised by Leyton's group in this Research Topic issue, this regulatory function of MSCs is correlated with high expression levels of Thy-1/CD90 (Leyton et al.).

Thy-1/CD90 is also expressed in fibroblasts, and Jiang and Rinkevich report that there are several populations of fibroblasts. These various cellular subsets are defined according to their anatomical site of origin and also on the basis of transient embryonic expression of genes, which program their ability to induce scar formation after wounding. In the skin, Thy-1/CD90 expression does not distinguish between fibroblasts with scarring ability and the other fibroblasts. In lung fibroblasts, the essential role in the regulation of mechano-transduction by integrin/Thy-1/CD90 interaction in response to increased or decreased matrix stiffness is reviewed by Hagood, while the same regulatory function in other cell types is reviewed by Hu and Barker. Additionally, in various inflammatory diseases, such as rheumatoid arthritis, systemic sclerosis, cancer, but not in cystic lung fibrosis, fibroblasts that overexpress Thy-1/CD90 have been related to disease progression (reviewed in Leyton et al.).

Contributing Authors point to some interesting facts and questions. For example, several years after providing the community with Thy-1/CD90 deficient mice (Nosten-Bertrand et al., 1996), Morris revised the influence of the genetic background on the phenotype. Sauzay et al. describe a possible role for the Unfolded Protein Response (UPR) in the regulation of Thy-1/CD90 expression. Other unanswered questions include, how the mode of anchorage of Thy-1/CD90 affects its signaling and function or the relevance of glycosylation in some of the seemingly discordant effects of Thy-1/CD90 in different contexts, or whether Thy-1/CD90 signaling has an effect on cell commitment.

In summary, Thy-1/CD90 functions as a microenvironment sensor that is important in pathological events, but the contributions of the molecule to disease development depend on the cellular context. Thus, using Thy-1/CD90 as a possible therapeutic target will require a better understanding of the positive and negative outcomes that inhibition of the molecule can lead to.

AUTHOR CONTRIBUTIONS

EF-B and LL were both guest editors of the Research Topic: Thy1/CD90 Surface Glycoprotein: Sensor of Microenvironment? and wrote this editorial.

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REFERENCES

Chen, Q., Shou, P., Zheng, C., Jiang, M., Cao, G., Yang, Q., et al. (2016). Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death Differ.* 23, 1128–1139. doi: 10.1038/cdd.2015.168

Estrela, C., Freitas Silva, B. S., Silva, J. A., Yamamoto-Silva, F. P., Pinto-Junior, D. D., and Gomez, R. S. (2017). Stem cell marker expression in persistent apical periodontitis. *J. Endod.* 43, 63–68. doi: 10.1016/j.joen.2016.09.002

Hermosilla, T., Munoz, D., Herrera-Molina, R., Valdivia, A., Munoz, N., Nham, S. U., et al. (2008). Direct Thy-1/$\alpha_V\beta_3$ integrin interaction mediates neuron to astrocyte communication. *Biochim. Biophys. Acta* 1783, 1111–1120. doi: 10.1016/j.bbamcr.2008.01.034

Lagos-Cabre, R., Alvarez, A., Kong, M., Burgos-Bravo, F., Cárdenas, A., Rojas-Mancilla, E., et al. (2017). $\alpha_V\beta_3$ Integrin regulates astrocyte reactivity. *J. Neuroinflammation* 14:194. doi: 10.1186/s12974-017-0968-5

Leyton, L., Schneider, P., Labra, C. V., Rüegg, C., Hetz, C. A., Quest, A. F., et al. (2001). Thy-1 binds to integrin beta(3) on astrocytes and triggers formation of focal contact sites. *Curr. Biol.* 11, 1028–1038. doi: 10.1016/s0960-9822(01)00262-7

Nosten-Bertrand, M., Errington, M. L., Murphy, K. P., Tokugawa, Y., Barboni, E., Kozlova, E, et al. (1996). Normal spatial learning despite regional inhibition of LTP in mice lacking Thy-1. *Nature* 379, 826–829. doi: 10.1038/379826a0

Picke, A. K., Campbell, G. M., Blüher, M., Krügel, U., Schmidt, F. N., Tsourdi, E., et al. (2018). Thy-1 (CD90) promotes bone formation and protects against obesity. *Sci. Transl. Med.* 10:eaao6806. doi: 10.1126/scitranslmed.aao6806

Sandhoff, R., Schulze, H., and Sandhoff, K. (2018). Ganglioside metabolism in health and disease. *Prog. Mol. Biol. Transl. Sci.* 156, 1–62. doi: 10.1016/bs.pmbts.2018.01.002

The Tabula Muris Consortium, Overall coordination, Logistical coordination, Organ collection and processing, Library preparation and sequencing, Computational data analysis, et al. (2018). Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature* 562, 367–372. doi: 10.1038/s41586-018-0590-4

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REVIEW
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Thy-1, a Pathfinder Protein for the Post-genomic Era

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Thy-1 is possibly the smallest of cell surface proteins – 110 amino acids folded into an Immunoglobulin variable domain, tethered to the outer leaflet of the cell surface membrane via just the two saturated fatty acids of its glycosylphosphatidylinositol (GPI) anchor. Yet Thy-1 is emerging as a key regulator of differentiation in cells of endodermal, mesodermal, and ectodermal origin, acting as both a ligand (for certain integrins and other receptors), and as a receptor, able to modulate signaling and hence differentiation in the Thy-1-expressing cell. This is an extraordinary diversity of molecular pathways to be controlled by a molecule that does not even cross the cell membrane. Here I review aspects of the cell biology of Thy-1, and studies of its role as deduced from gene knock-out studies, that suggest how this protein can participate in so many different signaling-related functions. While mechanisms differ in molecular detail, it appears overall that Thy-1 dampens down signaling to control function.

Keywords: membrane protein, GPI (glycosylphosphatidylinositol), adhesion, signaling system, mutant mice, neuron, astrocyte

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INTRODUCTION

When I last reviewed Thy-1 (Morris, 1992), we knew a lot about its chemical structure and its expression in neuronal and lymphoid tissues, and were just starting to glimpse its function (Tiveron et al., 1992). I titled that review "Thy-1, the Enigmatic Extrovert on the Neuronal Surface." Enigmatic, because whenever anyone looked carefully at Thy-1, they invariably found it awkward, refusing to do the expected and thereby revealing new mechanisms. And extrovert, because there simply is so much Thy-1 that it cannot be ignored, an abundance that led to its being the first chemically characterized mammalian membrane protein (apart from red blood cell proteins) (Williams et al., 1977), and a feature that surely is central to understanding its role.

Since then, neuronally expressed Thy-1 has been demonstrated to be an adhesive ligand for the integrin $\alpha_v\beta_3$ (Leyton et al., 2001; Hermosilla et al., 2008), inhibiting neurite extension from Thy-1 expressing neural cells and promoting focal adhesion formation, cell motility and inflammatory activation on the integrin-expressing partner, mature astrocytes (Hermosilla et al., 2008; Avalos et al., 2009; Herrera-Molina et al., 2012; Kong et al., 2013; Lagos-Cabre et al., 2017). Subsequently, additional integrins $\alpha_v\beta_5$ (Zhou et al., 2010), $\alpha_5\beta_1$ (Fiore et al., 2014), $\alpha_C\beta_2$ (Choi et al., 2005), and $\alpha_M\beta_2$ (Wetzel et al., 2004) have been shown to be Thy-1 receptors; syndecan-4, thrombospondin and sulphated glycans are frequently co-receptors in focal adhesions (Hueber et al., 1992; Rege et al., 2006; Avalos et al., 2009; Kong et al., 2013; Fiore et al., 2014); and CD97, a 7-transmembrane G-protein coupled adhesive receptor, has also been shown to be a Thy-1 receptor (Wandel et al., 2012), expanding the families of known Thy-1 receptors.

Each of these receptors activates Thy-1 to function as a differentiation-triggered switch in different tissues to control an increasingly diverse set of signaling pathways, either directly

[e.g., Cbp/Csk/Src-family kinases (Chen et al., 2009c; Maldonado et al., 2017), Fas (Cohen et al., 2009; Liu et al., 2017), PPARγ (Varisco et al., 2012), and the binding and uptake of mesenchymal stem cell derived extracellular vesicles (Shentu et al., 2017)]. Interdependence of Thy-1 with Wnt/β-catenin expression is emerging as an important regulator of bone and liver development (Cheng et al., 2014; Picke et al., 2018). Inappropriate expression of Thy-1 in these tissues affects oncogenesis, often (but not always) acting as a tumor suppressor (Kumar et al., 2016). Thy-1 usually acts in *trans*, binding to a receptor present on another cell and thereby regulating signaling in both cells (Herrera-Molina et al., 2013) but on a subset of lung fibroblasts it acts in *cis*, binding to the 'inactive' (bent) conformation of $\alpha_v\beta_3$ integrin on its own surface to act as a mechanosensitive detector (Fiore et al., 2015).

An important feature of activation of Thy-1 via its physiological receptors is that they act monovalently (e.g., soluble, monovalent $\alpha_v\beta_3$-Fc substitutes for the astrocytic receptor, inhibiting neurite outgrowth by neurons); cross-linking by a divalent receptor is not necessary (Herrera-Molina et al., 2012; Fiore et al., 2014; Maldonado et al., 2017). When protein A (PA) has been used to cross-link the Fc regions to produce divalent $(\alpha_v\beta_3\text{-Fc})_2$-PA, the response (neurite retraction; size of Thy-1 clusters) increased by ∼50% (Herrera-Molina et al., 2012) but this is be expected given that divalency squares the effective affinity ('avidity') of a membrane-bound ligand (Morris, 1994) and cross-linking would necessarily combine separate small clusters into bigger ones.

So, is Thy-1 still enigmatic? Very much so! That such a small protein can contribute to the fine control of such diverse cellular interactions is remarkable. Of course, now that we know that mankind has at most 22,000 genes (Abascal et al., 2018), just a few times more than the simplest bacterium, it follows that most mammalian proteins must be able to combine with other macromolecules (lipids, carbohydrates and nucleotides, as well as other proteins) to produce by combinatorial diversity the vast range of specialist structures and functions needed to create our extraordinarily complex bodies and minds. Current interest is focused on the ability of Intrinsically Disordered Proteins to adopt multiple conformations that allow them to form different functional complexes with different partners (e.g., Fu and Vendruscolo, 2015; Uversky, 2017; Berlow et al., 2018). As a single immunoglobulin domain stabilized by two internal disulphide bonds (Williams et al., 1977), Thy-1 is anything but intrinsically disordered. But it is able to influence, and be influenced by, its lipid environment, as I will argue here. Thy-1 is a prime exemplar of a membrane organizer for this post-genomic era: one little protein, modified with lipids and carbohydrates, that combines with multiple receptors and signaling pathways to fine tune diverse physiological functions.

Thy-1 ABUNDANCE – WHY SO MUCH?

Arguably the most detailed determination of the abundance of cell surface molecules for nucleated cells is for rat lymphocytes (Barclay et al., 1993). There are 10^6 molecules of Thy-1 per cell on rat thymocytes, amounting (in molar terms) to around 80% of the total cell surface protein, covering somewhere between 5 and 25% of the thymocyte surface at an average density of 7,100 molecules per μm^2. The next most abundant thymocyte protein is the large adhesive sialoglycoprotein CD43 (10^5 per thymocyte) (Barclay et al., 1993). The core signaling unit, the T Cell Receptor/CD3 complex, occurs at around 10^4 molecules per cell (Valitutti et al., 1995), as do co-receptor molecules CD4 and CD8 (Barclay et al., 1993). Thus, on thymocytes, Thy-1 is 100 times more abundant than mainstream signaling proteins, and 10x more abundant than a major adhesion protein. In the nervous system, the level of Thy-1 on axons is somewhat lower (500–1,500 molecules per μm^2; Beech et al., 1983) but this is still much higher than typical levels of expression on cell lines (e.g., 20 molecules of Thy-1 label per μm^2 on RBL-2H3 mast cells; Veatch et al., 2012). If Thy-1 is expressed in excess of any signaling or adhesive need, are there additional beneficial effects, for instance conferred by Thy-1's organization of its immediate lipid environment, that explain its high abundance?

DISTRIBUTION OF Thy-1 ON NAKED NEURONAL MEMBRANE

What does this abundant surface expression look like? **Figures 1A,B** show Thy-1 immunolabelling (Fab OX7 antibody fragments coupled directly to 40 nm gold) on axons growing in tissue culture from adult sensory neurons, viewed in a scanning electron microscope (SEM) by electron backscattering in which the gold appears as white spheres and the axons are evident only by their gold label (Madore et al., 1999). The 40 nm gold is huge compared to Thy-1, which has a 6 nm Stokes diameter (Barclay et al., 1975). Several Thy-1 molecules could underlie each gold particle. No detergent, necessary to expose Thy-1 in adhesion complexes to Fab antibodies, has been added, so this is a view only of the naked upper and lateral surfaces of the axons.

By any definition, this is clustered distribution of gold label: some axonal surface is heavily labeled, and some not at all. Clustering of surface molecules into nm domains is often the result of receptor activation. Could that be the case with Thy-1 on axons? For instance, could Thy-1 on the upper (non-adhesive) axonal surface already be bound in *cis* to receptors on that surface, as it does with $\alpha_v\beta_3$ to act as a mechanosensor on lung fibroblasts (Fiore et al., 2015)? Sensory neurons express a range of β1 integrins including α5β1 (Tomaselli et al., 1993) a known Thy-1 receptor (Fiore et al., 2014), as well as Syndecan-4 (Lin et al., 2015). Both are components of focal adhesions, and may affect Thy-1 distribution on the lower surface of the axons that adhere, in these cultures, to a laminin substrate. The *cis*-interaction of Thy-1 with $\alpha_v\beta_3$ appears to be specific to mechanosensing in the lung, and has been specifically excluded on neuronal cells (Maldonado et al., 2017). As far as we know, **Figure 1** shows examples of the distribution of unactivated Thy-1 on naked neuronal surface.

Figure 1C shows a higher power view of a large (23 gold labels) Thy-1 cluster seen in the Transmission Electron Microscope (TEM) of a section of axon labeled with monovalent Ox7

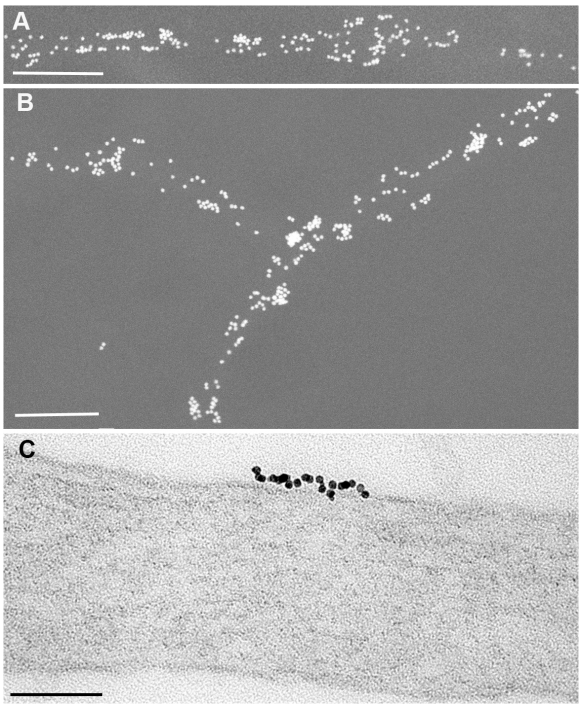

FIGURE 1 | Immunogold labeling of Thy-1 on axons of adult sensory neurons growing in culture, seen **(A,B)** as 40 nm Fab-gold viewed by electron backscattering in an SEM; and **(C)**, 5 nm Fab-gold seen in the TEM. In **(A,B)**, the culture was fixed for 20 min at 4°C with 0.5% paraformaldehyde + 0.5% glutaraldehyde, before washing and labeling with OX7 Fab coupled to 40 nm gold. In **(C)**, a live culture was labeled for 30 min at 4°C with monovalent 5 nm OX7 Fab gold before fixation as above and processing for TEM. Scale bars are 1 μm in **(A,B)** and 100 nm in **(C)**. From Madore et al. (1999) and Morris et al. (2004).

Fab-5nm gold, a label that is slightly smaller than Thy-1 and so able to label each Thy-1 molecule. Thy-1 is packed very tightly, with little if any room for additional interposed surface protein. This particular labeled patch is 125 nm in length, rather larger than the 10–20 nm range often identified as the size of membrane rafts of GPI-APs (Garcia-Parajo et al., 2014).

How reliable is our interpretation that the clustered gold label is an accurate reflection of the distribution of Thy-1 on the membrane? (Veatch et al., 2012) immunogold labeled unactivated IgE bound to FcεRI receptors on unstimulated RBL-2H3 mast cells, and obtained a distribution of 1-10 gold particles per patch. Extensive mathematical analysis and control experiments showed that their gold labeling was "dominated by multiple gold particles binding to single target proteins." Only when the IgE was activated, thereby activating its FcεRI receptor, did true receptor clustering occur. For their study, they used commercial divalent IgG antibodies raised against divalent Ig of other species. In my experience, one generally gets at least six molecules of secondary antibody binding to dimeric IgG primary antibody (that is, at least three epitopes on the 75 kDa monomer unit of IgG are recognized by anti-IgG antibody). The Stokes' diameters of IgG and IgE are 10.4 and 12.0 nm respectively (Griffiths and Gleich, 1972), providing enough distance from the FcεRI receptor to allow multiple 10 nm gold labels to bind, as found in this study. In contrast, we make our own anti-GPI-AP antibodies in house, digest them to monovalent Fab fragments which we directly couple to gold. Since 5 nm gold particles have a surface area of 78.6 nm^2, Fab (Stokes diameter 6.3 nm; Griffiths and Gleich, 1972) conjugates multiply to each 5 nm gold particle which thereby becomes multivalent. When we couple to gold we dilute the immune Fab with an excess of non-immune Fab until 30–50% of the gold does not bind at all to antigen and so contains no immune Fab, leaving the residual 50–70% predominantly labeled with 1 immune Fab to produce monovalent gold (see Supplementary Material, Sunyach et al., 2003). For Thy-1, we use monoclonal antibody OX7 directed to a single epitope centered on Arg 89 (Mason and Williams, 1980). Monovalent binding of gold to Thy-1 is built into our reagents. In addition to the live cell labeling of **Figure 1C**, to preclude any possible probe-induced movement of Thy-1 (or PrP^C), we also pre-fixed cells with fixative containing 0.5% EM grade glutaraldehyde, which covalently fixes proteins within msec, unlike paraformaldehyde that initially forms labile bonds that take several hours to covert to stable covalent bonds (Morris and Barber, 1983) allowing considerable post-fixed movement of GPI-AP (Mayor et al., 1994). The distribution of label we find with live or fixed tissue is indistinguishable by eye, and reflects the trouble we have taken to identify fixation conditions that immobilize each GPI-AP without destroying their epitopes recognized by our antibodies (Morris and Barber, 1983; Ford et al., 2002).

We estimated the diversity of size of Thy-1 clusters on cultured adult neurons in (Brügger et al., 2004) taking as the definition of a cluster, gold particles that were within 20 nm of each other. I have re-plotted that data in **Figure 2** to show the size distribution: 5.8% of label was solitary, a third in clusters of fewer than 5, and 50% of all label occurred in clusters of 8 or fewer. However, larger clusters were also observed. (Suzuki et al., 2012) note that the number of GPI-AP CD59 molecules per cluster, prior to ligand binding, rises to include clusters of > 10 CD59 when the level of surface expression is increased from 0.16 to 0.90 copies per μm, still three orders of magnitude below Thy-1's level on neurons. We used a

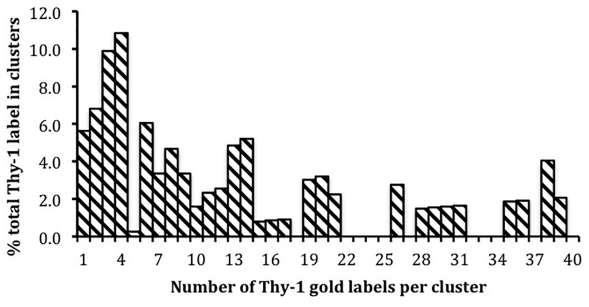

FIGURE 2 | Distribution of monovalent Thy-1 Fab-gold (10 nm) label in clusters containing different numbers of gold particles on the surface of cultured adult sensory neurons. Data from Brügger et al. (2004); 1881 gold particles in 387 clusters were counted.

crude definition of 'cluster' since our interest was not in the size of clustered Thy-1, but in whether it and PrP^C, a GPI-AP expressed on the same neuronal surface, just nm away from Thy-1 label, occupy the same, or identifiably different, membrane 'rafts.' At that time, membrane 'rafts' were treated as a single specialized lipid environment in which co-existed all GPI-APs expressed by the cell. Our demonstration (Brügger et al., 2004) that Thy-1 and PrP membrane domains could be isolated separately and had, reproducibly, different lipid compositions, was I believe the first indication that GPI-AP's on the same membrane tailored their lipids to suit their individual requirements, a point since made independently (Surviladze et al., 2007).

I suspect the greatest difference between our, and others', studies on GPI-AP is that we study endogenously expressed Thy-1 on primary cultures of mature neurons, having first acquired a detailed knowledge of the *in vivo* expression of the molecules (Morris, 1992; Ford et al., 2002). Standard cell lines are seductively convenient, but their ease of transfection compared to differentiated primary cells highlights their more porous membrane. Neuronal function is completely dependent upon the non-permeability of their surface membrane; and because neurons must sustain stability of their synaptic networks over our life-times, they are post-mitotic, very different from rapidly dividing cell lines, with vastly more intricate and longer-lived specialization of their cell surface. I question the reliance of so much work on cell lines to study 'rafts' as key organizers of cell surface function, when the cell lines have been selected for decades on the permeability of their membranes to transfection, and their high rate of uncontrolled cell division.

DISTRIBUTION OF Thy-1 ON NEURONAL MEMBRANE ADHERING TO ASTROCYTES

This topic is covered authoritatively elsewhere (Herrera-Molina et al., 2012; Maldonado et al., 2017). I draw your attention here to another recent study (Nemoto et al., 2017) because of the promise it shows in applying single molecule tracking to follow GPI-APs on the adherent surface of differentiating neurons in cultures. The cultures were of neonatal rat hippocampal neurons growing on astrocytes, at two points of postnatal differentiation (1 and 2 weeks in culture, when dendrites and axons are growing and synaptic networks start to form), observed at 37°C by TIRF microscopy focused on the substrate-adhesive surface membrane in contact with the underlying astrocytes (Nemoto et al., 2017). Individual molecules of PrP^C and Thy-1 exhibited identical overall behavior – long (seconds) periods of immobility interspersed with rapid diffusion. Such stalled periods are typical of GPI-APs that have formed a signaling complex that temporarily anchors the GPI-AP to the cytoskeleton (Suzuki et al., 2007a,b). The cell line CHO-K1, not of neuronal origin and not natively expressing Thy-1 (Suzuki et al., 2012) was used as a control.

The specific behavior of Thy-1 and PrP^C differed: for instance, around 50% of Thy-1 was immobilized at any time, compared to 71% of PrP^C; and the duration of Thy-1 mobile phases lasted more than twice that of PrP^C ((Nemoto et al., 2017), reproduced in **Table 1**). The tantalizing result in this study, however, was the duration of immobilized phases for both PrP^C and Thy-1, which showed exceptionally large standard deviations. For Thy-1, both means and standard deviations increased markedly during the 2nd week of growth; and for PrP^C, decreased. Technical problems limit the accuracy of the Thy-1 results (Nemoto et al., 2017) but Thy-1's rapidly increasing immobilization during neuronal maturation would be expected if it is binding to a variable but rising expression of its astrocytic $\alpha_v\beta_3$ receptor (Leyton et al., 2001; Herrera-Molina et al., 2012) which would immobilize Thy-1 on the adhesive substrate surface. The opposite, rapidly shortening immobilization of PrP^C could be due to an increasing expression of its endocytic partner, LRP1 (Parkyn et al., 2008) that rapidly recycles PrP^C on neurons every few minutes, leading it out of rafts and into coated pits, from where it is endocytosed then sorted in recycling endosomes and returned to neuronal surface rafts (Sunyach et al., 2003).

The application of single molecule, as well as super-resolution, fluorescent techniques to study Thy-1's action on the surface of cells that are interacting with their neighbors in complex primary cultures promises to add much to our understanding of molecular mechanisms of membrane function.

THE STRESS IMPOSED BY Thy-1 AND OTHER GPI-APs ON THEIR MEMBRANE LIPIDS

When Thy-1, embedded in a membrane, has its GPI-anchor cleaved by either phospholipase C or D, allowing the protein-glycosyl component to float free of the membrane, a large conformational change occurs on the opposite face of Thy-1, where Arg 89, that specifies the Thy-1.1 allele recognized by the OX7 monoclonal antibody, resides (Barboni et al., 1995). Despite OX7 being a particularly high affinity antibody (Mason and Williams, 1980), PLC/D cleavage of Thy-1 caused pre-bound OX7 to be released, and prevented any more antibody binding to its site. The conformational shift was evident in

TABLE 1 | Duration (photobleaching corrected) of MOBILE and IMMOBILE states, and % protein Immobilized at any time (in seconds), for Thy-1 and PrP^C on cultured hippocampal neurons at 37°C.

		Mobile Duration mean ± sem (s)	Immobile Duration mean ± sem, (s)	% Immobile GPI-AP mean ± sem
Neurons, 1 week	Thy-1	0.79 ± 0.21	4.11 ± 4.29	48.5 ± 8.2
	PrP	0.41 ± 0.049	4.69 ± 2.20	71.1 ± 3.4
Neurons, 2 weeks	Thy-1	0.99 ± 0.19	10.41 ± 15.02	54.8 ± 4.4
	PrP	0.38 ± 0.074	2.75 ± 1.43	71.8 ± 5.6
CHO-K1 cell line	Thy-1	1.91 ± 0.41	1.84 ± 0.65	51.7 ± 4.5
	PrP	0.75 ± 0.091	1.74 ± 0.36	71.4 ± 2.8

Data taken from Nemoto et al. (2017).

the Circular Dichroism spectrum of human Thy-1; and by Molecular Dynamics modeling of the effect of deacylation upon Thy-1 (Barboni et al., 1995). Similar conformational changes accompany deacylation of other membrane-bound GPI-APs (Butikofer et al., 2001; Paulick and Bertozzi, 2008; Bradley et al., 2013) suggesting the conformational effect of the membrane on the lipid-anchored protein is a general property of GPI-APs.

If the membrane can exert such strong conformational restraint upon Thy-1, then Newton's Third Law requires that the fully acylated protein exert an equal and opposite force upon the membrane. Is this an inconsequential curiosity, or is it telling us something functionally important about the interaction between GPI-AP's and their local membrane environment? While the raft membrane around GPI-APs is generally agreed to be in an ordered phase, the multiplicity of lipid species present in membranes allows multiple ordered phases to be formed (Brügger et al., 2004; Surviladze et al., 2007). There is no single lipid environment in 'rafts' – each protein tailors its lipids to suit its specific needs. Further, there is no single GPI-anchor – post-translational modification of the anchor is also tailored to the needs of individual proteins (Fujita and Kinoshita, 2012; Puig et al., 2014). And the phase of raft lipids is determined, not just by the ordered lipids, but also by the disordered, polyunsaturated lipids surrounding them (Bakht et al., 2007). Could the effect of the GPI-AP tension on its immediate 'raft' environment be unique to each GPI-AP and its expressing cell, and determine not only the conformation of the protein, but also the distinctive composition and phase properties of its surrounding lipids?

Any contribution to membrane tension conferred by GPI-APs will be altered by ligand binding, which in Thy-1's case on neurons may differ from general expectations (Garcia-Parajo et al., 2014). In the larger clusters (e.g., **Figure 1C**), Thy-1 molecules are closely adjacent, about 6 nm apart, each occupying ∼28 nm^2 (similar to the estimate of Suzuki et al., 2012). Integrins in focal adhesions occupy ∼100 nm^2 (Brinkerhoff and Linderman, 2005), giving an equivalent diameter of ∼11.2 nm. On astrocytes, the spatial distribution of $\alpha_v b_3$ integrins will presumably force bound Thy-1 to spread out to fourfold lower density than in a non-activated cluster.

Further, ordered domains formed in the outer leaflet induce complementary ordered domains in the apposed inner leaflet (Allender and Schick, 2006; Collins and Keller, 2008; Kiessling et al., 2009; Raghupathy et al., 2015). Should each GPI-AP imprint its distinctive presence on its outer leaflet lipids, do they in turn pass that imprint on, to selectively recruit specific inner leaflet lipids to their raft? The interactive composition of the ordered domain in both leaflets could determine which of available transmembrane adaptor proteins (e.g., LAT, PAG/Cbp, NTAL and LIME; Horejsi et al., 2004) dock with Thy-1. These adaptor proteins have minimal (a few amino acids) extracellular domains, and functionally require palmitoylation of Cys residues located at the cytoplasmic end of their transmembrane domain that interact with the inner leaflet of raft membrane (Tanimura et al., 2006). Inner, as well as outer, lipid leaflets of a raft are involved in establishing the appropriate environment for interaction with signaling complexes.

Since the outer leaflet influences the conformation of GPI-APs, does the inner leaflet similarly impose conformational constraints upon cytoplasmic diacylated proteins such as the Src Family Kinases (SFK)? Cytoplasmic diacylation attaches C14/C16 or C16/C16 saturated lipids directly to the N-terminal amino acids, with no glycosyl linker (van't Hof and Resh, 2000). A membrane effect upon protein conformation could be even stronger for these inner leaflet proteins. Does protein/lipid tension operate on both sides of the surface membrane?

Interest in membrane tension has primarily focused upon large scale parameters such as membrane curvature and line tension (e.g., Huttner and Zimmerberg, 2001; Kuzmin et al., 2005), but is now, in studying the mechanism of signaling by the T Cell Receptor (TCR), moving to direct mechanosensor effects on protein conformation that allow 'catch' and 'slip' bonds to differentiate between activation by foreign, or self, antigen (Chakraborty and Weiss, 2014; Liu et al., 2014; Sibener et al., 2018). The precise (Å scale) positioning of different proteins of the signaling complex is also emerging as a key differentiator of the course of TCR signaling (Chakraborty and Weiss, 2014). A similar tension-dependent catch-and-slip mechanism has been found for the co-operative binding of Thy-1 by $\alpha_5\beta_1$ integrin and Syndecan-4 in contractility-dependent mechanosignalling of melanoma cells (Fiore et al., 2014). Could tension between membrane proteins and lipids be a factor here?

These speculative questions reflect my view that we have over-simplified our analysis of membrane lipids and proteins; we need better assay systems and finer grain analysis to fully understand membrane mechanisms. Membrane lipids are generally considered in overall classes (e.g., saturated vs. unsaturated; glycerolipids vs. sphingolipids), and similar members of the same class can undoubtedly substitute for each other in some aspects of membrane function. However, it is now clear that highly specified individual membrane lipids (e.g., C18:0/20:4 phosphatidylserine, and not other phosphatidylserines) are critical for individual steps in cytokinesis (Atilla-Gokcumen et al., 2014). More generally, individual lipids are being shown to be required at different stages in a cell's biology (Storck et al., 2018). Specific lipids in both leaflets of the membrane marshaled by individual GPI-APs could be functionally important in the transfer of information across the surface membrane. While the variety of receptors with which Thy-1 interacts is controlled by binding sites on Thy-1's protein domain, the precise lipids that surround Thy-1, which could differ in different cells and states of differentiation, could help select the transmembrane signaling mechanism that is addressed.

ISOLATION OF LIPID 'RAFTS' AS DETERGENT RESISTANT MEMBRANE (DRM)

It clearly would be a huge advance if individual rafts could be isolated and their full range of components identified. Endless experiments have sought to do just this, particularly using the resistance to solubilization by mild detergents of cholesterol-condensed saturated lipids to isolate rafts as DRM.

Significant opinion holds that rafts cannot be isolated – they are transient, critically temperature dependent (Honerkamp-Smith et al., 2008) assemblies whose defining characteristics are destroyed in the instant they are exposed to detergent (e.g., Munro, 2003). This view arises from the repeated demonstration by single molecule tracking that individual raft molecules are highly mobile; and even when immobilized as part of a signaling complex, they remain so for seconds at most (e.g., Shibata et al., 2012; Nemoto et al., 2017; Suzuki et al., 2007a,b, 2012, 2018). However rapid the motility of individual components, the individual raft can be considerably longer lived; for instance, integrins continuously enter and leave stationary focal adhesions (Shibata et al., 2012; Tsunoyama et al., 2018). Fast turnover of individual components allows continuous fine tuning of the strength and duration of signaling, that can persist for minutes while being modified on a second by second basis (Suzuki et al., 2007a; Tian et al., 2007; Harding and Hancock, 2008).

An alternative case for dismissing DRMs comes from studies of the mechanism of detergent solubilization of model bilayers, where the detergent induces the artefactual formation of subdomains in the membrane (Heerklotz, 2002; Heerklotz et al., 2003; Lichtenberg et al., 2005, 2013). However, models are proving poor substitutes for real cell membranes (Lee et al., 2015). More tellingly from a physicist's viewpoint, since the classical Lo phase is remarkably temperature stable (Ipsen et al., 1987) as are the surface membranes of cells (Lee et al., 2015), it must be possible to isolate DRMs at physiological temperature if they truly are derived from membrane rafts.

Using the standard method for DRM isolation, we and others have shown that the DRMs obtained are fusions of totally different membranes that do not preserve the outer/inner leaflet distinction of *in vivo* membranes, and indeed contain very few inner leaflet lipids; they are several μm in size, orders of magnitude greater than any raft is thought to be (Madore et al., 1999; Morris, 2010).

We also, using appropriate solubilization conditions, have immunoaffinity isolated separately Thy-1 and PrP^C from brain membrane DRMs (Madore et al., 1999; Brügger et al., 2004). Each DRM had, very reproducibly, distinctly different lipid compositions (Brügger et al., 2004). Similarly Thy-1 and FcεRI, again located in near-adjacent but separate clusters on the mast cell surface as seen by SEM (Veatch et al., 2012) have been immuno-isolated as separate DRMs, again with different lipid compositions (Surviladze et al., 2007). And, most remarkable of all, (Han et al., 2009) used bulk-isolated DRMs from FcεRI-activated mast cells to follow the temporal sequence of activation from ligand binding to receptor engagement with the actin cytoskeleton.

We reasoned that the critical difference in the methods used lies in the ions present, including Ca^{2+} released by ruptured cells. Not only are bilayer lipids asymmetrically distributed (Morris, 2010); the cations Ca^{2+} and Na^+ bathe the outer leaflet, Mg^{2+} and K^+ bathe the inner leaflet. These monovalent cations contribute significantly to the bilayer strength of individual lipids (Garcia-Manyes et al., 2005, 2010; Beedle et al., 2015). The most critical effect is the release of Ca^{2+} from solubilized cells upon the matrix of Phosphatidyl Serine (PS^-) interleaved with Phosphatidyl Ethanolamine (PE) to form the inner leaflet. Ca^{2+}, gaining access to the inner leaflet, chelates the negatively charged PS^-, thereby withdrawing it from the PE lattice, which thus destabilized (Cullis and Verkleij, 1979; Tilcock and Cullis, 1981; Tokutomi et al., 1981; Bally et al., 1983) would be quickly removed by the detergent leaving monolayer fragments of outer membranes that fuse, thus producing the artifacts mentioned above (Morris, 2010).

We therefore used for solubilization an 'intracellular' buffer containing EGTA to mop up Ca^{2+} as it is released from cells; and used concentrations of K^+, Mg^{2+} and acetate to mimic the intracellular environment. We could isolate DRMs at 37°C (and even at 55°C) that were a distinct improvement over isolation with other buffers at 4°C. Specifically, the 37°C DRMs had a more balanced proportion of inner and outer membrane lipids; Thy-1 and PrP^C were isolated completely separately; intracellular 'raft' components, such as Fyn and Flotillin were more completely recovered in the isolated DRMs; and actin (which we have never previously seen in any DRM), was isolated with Thy-1 but not PrP^C DRMs (Chen et al., 2009a,b). Src kinase, of particular relevance to Thy-1 signaling (Chen et al., 2009c; Maldonado et al., 2017) is untypical within the Src family of kinases (SFK) in being anchored to the inner membrane surface, not by two diacyl chains, but by a myristate plus basic protein patch (van't Hof and Resh, 2000). The positively charged patch binds to negatively charged phosphatidylinositol patches on the inner surface membrane, linking the kinase to the membrane. Only when using the intracellular buffer have we seen substantial Src co-isolate with DRMs in the light fractions of a sucrose gradient at any temperature, much less at 37°C (Chen et al., 2009b).

In these studies we compared the benchmark DRM detergent, Triton X100, with Brij 96, which differ in the hydrophobic tail of Triton X-100 being short, disrupting primarily the interfacial region and outer leaflet, whereas Brij 96 has a longer, bilayer-spanning hydrophobic tail. Overall, Brij 96 gave a better yield of proteins and lipids in the DRM fractions, but these were marginal compared to the use of intracellular buffer (Chen et al., 2009a,b).

Some salient features of detergent solubilization of brain membranes, using 0.5% Brij 96 at 37°C with the intracellular buffer, are shown in **Figure 3** (Morris et al., 2011). The top panel (**Figures 3A–C**) shows membranes that remain in the bottom (highest density) fraction after sucrose gradient fractionation. Remarkably, synaptic junctions with clearly identifiable pre- and post-synaptic components, and mitochondrial inner membrane, are preserved in this buffer. Although not sufficiently lipid-rich/protein-sparse to float in the low density fractions on the gradient, major functional elements of brain retain recognizable structure under these solubilization conditions, indicating that detergent resistant membrane is a much more inclusive category than just low density DRMs. It is the low protein:lipid ratio of DRMs that makes them float with the lipids, rather than be retained in the high density fractions of the gradient.

Figures 3D,E show examples of DRMs isolated at low density after overnight centrifugation on the sucrose gradient. Many of the vesicles remain large, and only small sectors of them label for either Thy-1 or PrP^C (Chen et al., 2009b). This suggests that membrane fusion of Thy-1 and PrP^C DRMs with other DRMs

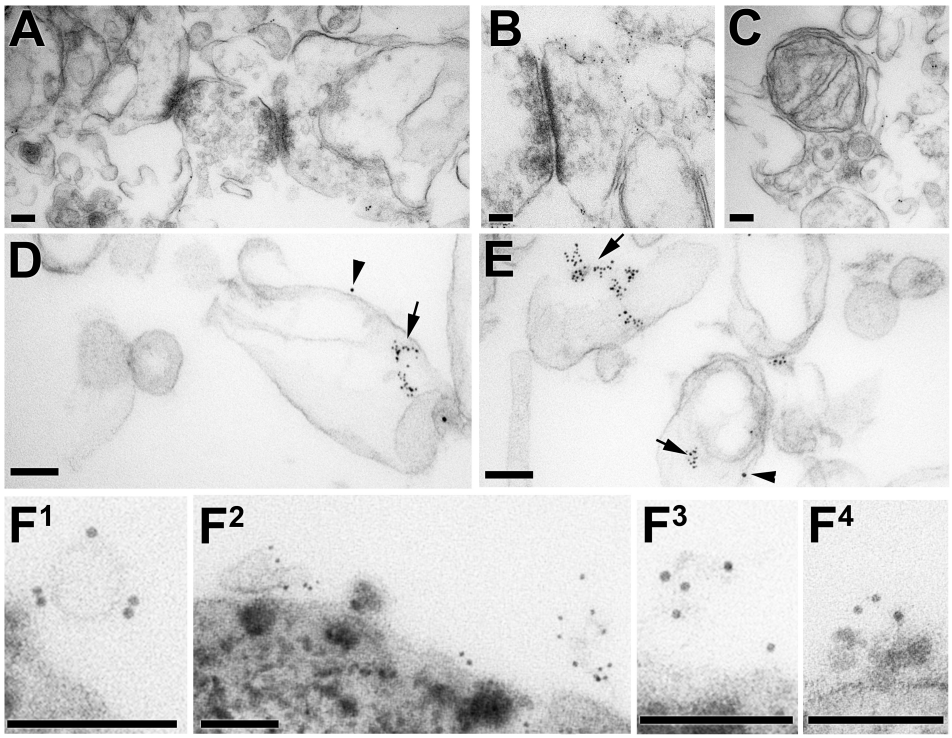

FIGURE 3 | TEM photographs documenting progress in isolating Thy-1 and PrP^C in membrane fragments of a size and composition expected of 'rafts,' after solubilization of brain membranes for 15 min at 37°C in Brij 96 with intracellular buffer (Chen et al., 2009b; Morris et al., 2011). **(A–E)** Show samples recovered from a sucrose gradient; **(F^1–F^4)** are samples recovered immediately after solubilization (without the density gradient step) by their binding to anti-Thy-1 magnetic beads. Scale bars throughout are 100 nm. **(A–C)** Are detergent-resistant membranes recovered in the high density sucrose at the bottom of the gradient, with synaptic junctions recognizable in **(A,B)** by the post-synaptic densities; some pre-synaptic vesicles also remain. A remarkably intact mitochondrion in seen in **(C)**. **(D,E)** Show DRMs isolated from the light sucrose density fraction of the same gradient after centrifugation at 200,000 *g* for 18 h, immunolabelled for PrP^C (5 nm gold, arrows) and Thy-1 (10 nm gold, arrowheads). The DRMs are near μm in size, and the immunolabelled areas are a very small fraction of the whole vesicle. **(F^1–F^4)** Show brain membranes solubilized in Brij 96 in intracellular buffer at 37°C, with magnetic beads labeled with OX7 anti-Thy-1 IgG added after 5 min. At 15 min the beads were collected magnetically, washed and incubated with 5 nm gold labeled with OX7 Fab. The large heterogeneous objects at the bottom of **(F1–F^4)** are sections of magnetic beads.

containing neither of these two GPI-APs was occurring, either during solubilization or during the 18 h ultracentrifugation step that specifically concentrates the light DRMs.

We therefore reduced the entire procedure to just 15 min – 5 min solubilization followed by 10 min immunoaffinity isolation on magnetic beads, all at 37°C, which yielded 20–50 nm membrane fragments bound to the magnetic beads, finally of the size expected of membrane 'rafts' (**Figures 3F^1–F^4**).

Circumstances forced me to close down my lab shortly after this paper was submitted, so I have not progressed this research. But for anyone with an in-house supply of relevant antibodies, I suggest adapting this approach to your material is likely to give you access to raft membrane.

ANALYSIS OF Thy-$1^{-/-}$ (KNOCKOUT) MICE: THE FUNCTION OF Thy-1 *IN VIVO*

We produced and analyzed Thy-$1^{-/-}$ mice in the heady days of the '90's when careers were launched, or aborted, on the post-doc's ability to find an interesting behavioral phenotype in the analysis of mice in which they had inactivated (knocked out) a specific gene. Everyone was using the same embryonic stem cells derived originally from a 129/Sv/Ev strain mouse. The phenotype most commonly reported was excessive aggression.

Our mice too were remarkably aggressive. Our male littermates, if housed in the same cage, killed each other. I placed an experienced wild type (Thy-$1^{+/+}$) male into a cage with two Thy-$1^{-/-}$ females to initiate breeding, which usually started very quickly. Within a minute the male was clinging to the bars on the top of the cage, squealing to be let out. We outbred the mice, and within a few generations had separated the Thy-$1^{-/-}$ gene from the aggression-inciting gene. We continued this outbreeding on the 129/Sv/Ev and C57Bl6 backgrounds; and we derived an entirely independent Thy-1 knockout in C57Bl6 embryonic stem cells, and bred both onto C57Bl6 and 129/Sv/Ev backgrounds, to obtain genetically diverse but stable strains for behavioral analysis (Mayeux-Portas et al., 2000).

I mention this because scientific standards in this burgeoning field were initially not uniformly sound. We pointed out this problem in a Letter to Trends in Neuroscience (Morris and

Nosten-Bertrand, 1996), which elicited a phone call from a Nature editor lamenting my failure to send it to them, as they strongly suspected there was an underlying problem with these recurrent findings of aggression. An interesting Banbury Conference on Genetic Background in Mice in 1997 laid down standards in the field (Conference, 1997). You should not believe everything you read about knockout mice of the era; but our mice provide a sound platform for analysis, and have been used in some impressive recent studies (Zhou et al., 2004; Varisco et al., 2012; Cohen et al., 2018; Picke et al., 2018).

Lymphoid Phenotype

The 129/Sv/Ev mice were analyzed by He's lab studying the development of thymocytes into T cells (Hueber et al., 1997). Overall, they conclude that Thy-1 negatively regulates TCR-mediated signaling to fine-tune activation thresholds during thymocyte differentiation. In particular, transition from CD4/CD8 'double positive' thymocytes to mature single positive T lymphocytes was reduced; and a significant proportion of older Thy-$1^{-/-}$ mice (from both 129Sv/Ev and C57Bl6 backgrounds) developed T lymphomas. The latter is documented in their Supplementary Material, which also shows TEM photographs in which adhesion between thymocytes and the thymic epithelium is defective in Thy-$1^{-/-}$ mice, evident as gaps between the cells that are in sharp contrast to the firm intercellular adhesion normally found (Hueber et al., 1997). This confirmed earlier work (He et al., 1991; Hueber et al., 1992) that Thy-1 binds thymocytes to thymic epithelia. The occurrence of T lymphomas in older mice was, I believe, the first indication that Thy-1 is a tumor suppressor.

These studies of thymocyte differentiation rely upon the *in vivo* action of Thy-1 interacting with its receptors and signaling pathways. He's group also examined the molecular events following activation of the T-cell receptor (TCR) signaling in isolated thymocytes (Hueber et al., 1997). Isolation of thymocytes as a single cell suspension, away from their surrounding stromal cells, presumably removes integrin binding to Thy-1. They found the initial phosphorylation steps in the kinase pathways activated by TCR engagement (phosphorylation of CD3ϵ, TCRζ, and Lck; Ca^{2+} mobilization) occurred in Thy-$1^{-/-}$ thymocytes with around twice the intensity of the Thy-$1^{+/+}$ controls, resulting in thymocyte proliferation at twice the rate in Thy-$1^{-/-}$ compared to Thy-$1^{+/+}$ thymocytes. Thus Thy-1 not only lowers TCR signaling; it apparently does so in the absence of its integrin receptor, although whether to the same extent is not known.

Beissert et al. (1998) went on to study cutaneous T cell function in the Thy-$1^{-/-}$ mice, both *in vivo* and in culture. They found lower hypersensitivity responses in the Thy-$1^{-/-}$ mice, but otherwise relatively normal T cell function. However, the mechanism was the opposite to that of thymocytes. Isolated Thy-$1^{-/-}$ T cells, when stimulated via CD3 antibodies, showed lower activation of SFK and reduced mobilization of intracellular Ca^{2+}. The net effect of constitutive Thy-1 loss was reduced overall function, but by opposite changes in SFK phosphorylation and Ca^{2+} levels in thymocytes and their T cell progeny. The outcome of TCR activation in the two cell types is very different – progression into different differentiation pathways for thymocytes, mounting a final effector function (hypersensitivity response) for T lymphocytes. Their TCR-activated intracellular signaling pathways must diverge to produce different outcomes. Mouse T lymphocytes have only 10% the Thy-1 levels of thymocytes (Acton et al., 1974); could Thy-1's regulation of signaling when expressed at low levels, differ from that at high levels?

Neural Phenotype

Our extensive studies of the appearance during development of Thy-1 mRNA and protein in rodent nervous system showed that *in vivo*, Thy-1 is solely expressed on neurons, and exhibited extraordinary post-transcriptional regulation of expression, the protein being kept away from growing tips of dendrites, and excluded from axons until they had synapsed on their target neurons and stopped growing. Once expressed, Thy-1 then remained as a major component of the neuronal membrane throughout adult life, reviewed in Morris (1992). The exclusion of Thy-1 protein from growing axons, so prominent *in vivo*, is not found in primary tissue culture where expression of Thy-1 mRNA rapidly leads to expression of its protein (Morris, 1992).

We therefore looked for Thy-1-dependent function in adult brain, where, in a healthy individual, a fine balance is maintained between excitatory circuits and local inhibitory neurons that allows excitatory circuits to fire, modulated by inhibitory synapses that refine, restrict and finally return the circuit to its resting potential. Failure to terminate firing of excitatory circuits can lead to recycling pulses of excitation seen as seizures.

Electrophysiological studies with Thy-$1^{-/-}$ mice focused on the hippocampus and its associated dentate gyrus (Nosten-Bertrand et al., 1996; Errington et al., 1997; Hollrigel et al., 1998). The synaptic response of NMDA glutamate receptors at excitatory synapses of both large projection neurons (CA1 pyramidal cells) and smaller local neurons (dentate granule cells) were identical for Thy-$1^{+/+}$ and Thy-$1^{-/-}$ mice, except in their ability to convert persistent (tetanic) signaling into long-term enhanced responsiveness of affected synapses, called Long Term Potentiation (LTP) (Bliss et al., 2018). LTP was induced normally in the CA1 pyramidal cells, showing that basic excitatory signaling was functioning correctly, and complex long-term adaptation also progressed normally. However, similar NMDA glutamate receptor excitation of dentate granule interneurons failed, in anesthetized mice, to induce LTP. This could be overcome by adding in the recording pipette a pharmacological antagonist (bicuculline) to the inhibitory $GABA_A$ receptor. Bicuculline prevents the action of local inhibitory input on the recorded cell, and with this inhibition removed, the dentate granule cells developed LTP when appropriately stimulated at their excitatory NMDA receptors. Subsequent studies on the inhibitory input to the dentate granule cells in Thy-$1^{-/-}$ mice showed them to be identical to those of Thy-$1^{+/+}$ mice in most respects, except that in Thy-$1^{-/-}$ mice, spontaneous inhibitory post-synaptic currents were stronger, especially if two action

potentials arrived in quick (msec) succession (Hollrigel et al., 1998).

Thus, in the hippocampus (a good model for most of the brain), the absence of Thy-1 causes too strong a response, not in the main excitatory circuitry, but in the inhibition of these circuits by local inhibitory neurons. Thy-1's action is pre-synaptic, down-regulating inhibitory vesicle number, size, content or release, mechanisms primarily controlled by influxes of Ca^{2+} (Sudhof, 2013) rather than kinases.

Confirmation of this mode of action in the brain could be tested once a clear behavioral phenotype became evident for Thy-$1^{-/-}$ mice (Mayeux-Portas et al., 2000). Thy-$1^{-/-}$ mice of both genotypes failed the test of 'social transmission of food preference,' yet performed as well as normal mice when they were injected with a high concentration (15 mg/kg) of a $GABA_A$ antagonist (pentylenetetrazole). This dose is sufficient to take a normal mouse to the verge of developing epileptic-type seizures, yet it enabled the Thy-$1^{-/-}$ mice to be 'normal.' In the absence of Thy-1, the $GABA_A$ inhibitory network is overly strong, approximately twice the level of normal Thy-$1^{+/+}$ mice.

It follows that raising Thy-1 levels should weaken inhibition and make mice more prone to seizure. As it happened, we had derived a mouse line that over-expressed Thy-1, and used them in this study for transgenic rescue of the Thy-$1^{-/-}$ mice (Mayeux-Portas et al., 2000) until a sustained fire alarm went off and a loud bell in the animal house sounded the evacuation for about 30 min. When we returned, all the Thy-1 over-expressing mice were dead, apparently from audiogenic seizure. This was not an experiment we would choose to do, but circumstances intervened to do so.

ON THE SOCIAL LIFE OF MICE....

To find a behavioral phenotype exhibited by Thy-$1^{-/-}$ mice, we looked for normal mouse behavior whose disruption would endanger the existence of mice as a species. Olfaction is the most important sensory input for mice; olfactory memory is established via the dentate gyrus and hippocampus. We screened for olfactory memory tasks, of which the 'social transmission of scented food' tests a species-critical strength of murine social behavior. When a mouse leaves its burrow to forage, it will preferably eat food it knows from experience is safe; but if a new berry or seed has appeared, it may be tempted to taste it. On returning to its burrow, looking well fed and happy, the other mice gather around and smell its breath to identify what it has been eating. If they detect a new odor, and the mouse is still looking healthy, then when they go out to forage they will eat that berry or seed. Thus knowledge of what is safe to eat, and what is not, is passed around the colony. This highly evolved social behavior plays a significant role in the success of the species, and so would be maintained by evolutionary pressure. The Thy-$1^{-/-}$ mice could be readily trained to use olfactory clues to follow a trail to food (they could smell and had olfactory-based memory); they spent as long as normal mice smelling the 'demonstrator' mouse when it was returned to the cage having eaten food emitting a new scent. It was only in using the information about the scented food carried on the breath of the 'demonstrator' mouse, to then choose which food to eat, that the Thy-$1^{-/-}$ mice were defective. We have no reason to think this is the only phenotype displayed by these mice. Thy-$1^{-/-}$ mice, for instance, were more flexible in searching for a new position of the hidden platform in the watermaze, which was significant in the 129/Sv/Ev but not C57Bl6 backgrounds (Mayeux-Portas et al., 2000).

CONCLUSION

I started this review calling Thy-1 a pathfinder protein for the post-genomic era, because its domains (protein, lipid and two types of carbohydrate chain) confer upon it exceptional functionality that is used to regulate a diverse range of developmental decisions. In the brain, the demonstration by Leyton and colleagues of the inter-dependence of neuronal Thy-1 and astrocytic integrins is timely given the growing realization that the astrocyte is not just a general support cell, but rather is an active partner with neurons in controlling the formation and subsequent modification of synapses via contact mediated signals (Clarke and Barres, 2013). For the mature function of the nervous system, synapses must remain adaptable within relatively stable neuronal networks. I believe the effect of Thy-1 is to calm down growth signals in neurons to enable a relatively stable synaptic framework to be maintained, while promoting astrocytic growth as the brain expands during postnatal maturation leading into adulthood.

Elsewhere, Thy-1 is expressed as a molecule that decides cell fate in the development of various tissues, and limits growth to be normal, not oncogenic.

There has been excellent progress in the past decade, celebrated in this Frontiers issue, filling out these generalities with precise mechanistic details of protein-to-protein interactions enabled by heterogeneity in the membrane environment. But there remain puzzles, such as why Thy-$1^{-/-}$ lymphocytes, isolated from their stromal cells and so from possible receptors, retain altered SFK signaling and Ca^{2+} mobilization. The stress observed exerted by the membrane on the conformation of Thy-1 is particularly intriguing, and possibly far-reaching in its implications.

In the coming decade, I think the Thy-1 membrane environment will emerge from anonymity, not just a 'raft' but as a highly defined, individual patch of membrane that actively enables Thy-1 to be involved in such a range of functions. In a world of Intrinsically Disordered Proteins, Individually Ordered Lipids will have their day.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

REFERENCES

Abascal, F., Juan, D., Jungreis, I., Martinez, L., Rigau, M., Manuel Rodríguez, J., et al. (2018). Loose ends: almost one in five human genes still have unresolved coding status. *Nucleic Acids Res.* 46, 7070–7084. doi: 10.1093/nar/gky587

Acton, R. T., Morris, R. J., and Williams, A. F. (1974). Estimation of the amount and tissue distribution of the rat Thy-1.1 antigen. *Eur. J. Immunol.* 4, 598–602. doi: 10.1002/eji.1830040904

Allender, D. W., and Schick, M. (2006). Phase separation in bilayer lipid membranes: effects on the inner leaf due to coupling to the outer leaf. *Biophys. J.* 91, 2928–2935. doi: 10.1529/biophysj.106.086868

Atilla-Gokcumen, G. E., Muro, E., Relat-Goberna, J., Sasse, S., Bedigian, A., Coughlin, M. L., et al. (2014). Dividing cells regulate their lipid composition and localization. *Cell* 156, 428–439. doi: 10.1016/j.cell.2013.12.015

Avalos, A. M., Valdivia, A. D., Munoz, N., Herrera-Molina, R., Tapia, J. C., Chiong, M., et al. (2009). Neuronal Thy-1 induces astrocyte adhesion by engaging syndecan-4 in a cooperative interaction with alphavbeta3 integrin that activates PKCalpha and RhoA. *J. Cell Sci.* 122, 3462–3471. doi: 10.1242/jcs.034827

Bakht, O., Pathak, P., and London, E. (2007). Effect of the structure of lipids favoring disordered domain formation on the stability of cholesterol-containing ordered domains (lipid rafts): identification of multiple raft-stabilization mechanisms. *Biophys. J.* 93, 4307–4318. doi: 10.1529/biophysj.107.114967

Bally, M. B., Tilcock, C. P., Hope, M. J., and Cullis, P. R. (1983). Polymorphism of phosphatidylethanolamine-phosphatidylserine model systems: influence of cholesterol and Mg2+ on Ca2+-triggered bilayer to hexagonal (HII) transitions. *Can. J. Biochem. Cell Biol.* 61, 346–352. doi: 10.1139/o83-048

Barboni, E., Pliego Rivero, B., George, A. J. T., Martin, S. R., Renouf, D. V., Hounsell, E. F., et al. (1995). The glycophosphatidylinositol anchor affects the conformation of Thy-1 protein. *J. Cell Sci.* 108, 487–497.

Barclay, A. N., Birkeland, M. L., Brown, M. H., Beyers, A. D., Davis, S. J., Somoza, C., et al. (1993). *The Leukocyte Antigen FactsBook*. London: Academic Press, 424.

Barclay, A. N., Letarte-Muirhead, M., and Williams, A. F. (1975). Purification of the Thy-1 molecule from rat brain. *Biochem. J.* 151, 699–706. doi: 10.1042/bj1510699

Beech, J. N., Morris, R. J., and Raisman, G. (1983). Density of Thy-1 on axonal membrane of different rat nerves. *J. Neurochem.* 41, 411–417. doi: 10.1111/j.1471-4159.1983.tb04757.x

Beedle, A. E., Lezamiz, A., Stirnemann, G., and Garcia-Manyes, S. (2015). The mechanochemistry of copper reports on the directionality of unfolding in model cupredoxin proteins. *Nat. Commun.* 6:7894. doi: 10.1038/ncomms8894

Beissert, S., He, H. T., Hueber, A. O., Lellouch, A. C., Metze, D., Mehling, A., et al. (1998). Impaired cutaneous immune responses in Thy-1-deficient mice. *J. Immunol.* 161, 5296–5302.

Berlow, R. B., Dyson, H. J., and Wright, P. E. (2018). Expanding the paradigm: intrinsically disordered proteins and allosteric regulation. *J. Mol. Biol.* 430, 2309–2320. doi: 10.1016/j.jmb.2018.04.003

Bliss, T. V. P., Collingridge, G. L., Morris, R. G. M., and Reymann, K. G. (2018). Long-term potentiation in the hippocampus: discovery, mechanisms and function. *Neuroforum* 24, A103–A120. doi: 10.1515/nf-2017-A059

Bradley, J. E., Chan, J. M., and Hagood, J. S. (2013). Effect of the GPI anchor of human Thy-1 on antibody recognition and function. *Lab. Invest.* 93, 365–374. doi: 10.1038/labinvest.2012.178

Brinkerhoff, C. J., and Linderman, J. J. (2005). Integrin dimerization and ligand organization: key components in integrin clustering for cell adhesion. *Tissue Eng.* 11, 865–876. doi: 10.1089/ten.2005.11.865

Brügger, B., Graham, C. H., Leibrecht, I., Mombelli, E., Jen, A., Wieland, F. T., et al. (2004). The membrane domains occupied by glycosylphosphatidylinositol-anchored prion protein and Thy-1 differ in lipid composition. *J. Biol. Chem.* 279, 7530–7536. doi: 10.1074/jbc.M310207200

Butikofer, P., Malherbe, T., Boschung, M., and Roditi, I. (2001). GPI-anchored proteins: now you see 'em, now you don't. *FASEB J.* 15, 545–548. doi: 10.1096/fj.00-0415hyp

Chakraborty, A. K., and Weiss, A. (2014). Insights into the initiation of TCR signaling. *Nat. Immunol.* 15, 798–807. doi: 10.1038/ni.2940

Chen, X., Jayne Lawrence, M., Barlow, D. J., Morris, R. J., Heenan, R. K., and Quinn, P. J. (2009a). The structure of detergent-resistant membrane vesicles from rat brain cells. *Biochim. Biophys. Acta* 1788, 477–483. doi: 10.1016/j.bbamem.2008.11.023

Chen, X., Jen, A., Warley, A., Lawrence, M. J., Quinn, P. J., and Morris, R. J. (2009b). Isolation at physiological temperature of detergent-resistant membranes with properties expected of lipid rafts: the influence of buffer composition. *Biochem. J.* 417, 525–533. doi: 10.1042/BJ20081385

Chen, Y., Veracini, L., Benistant, C., and Jacobson, K. (2009c). The transmembrane protein CBP plays a role in transiently anchoring small clusters of Thy-1, a GPI-anchored protein, to the cytoskeleton. *J. Cell Sci.* 122, 3966–3972. doi: 10.1242/jcs.049346

Cheng, B. Q., Jiang, Y., Zhu, Q., and Lin, W. G. (2014). Wnt/beta-catenin aids in regulating the proliferation of hepG2 cells mediated by thy-1. *Genet. Mol. Res.* 13, 5115–5127. doi: 10.4238/2014.July.7.4

Choi, J., Leyton, L., and Nham, S. U. (2005). Characterization of alphaX I-domain binding to Thy-1. *Biochem. Biophys. Res. Commun.* 331, 557–561. doi: 10.1016/j.bbrc.2005.04.006

Clarke, L. E., and Barres, B. A. (2013). Emerging roles of astrocytes in neural circuit development. *Nat. Rev. Neurosci.* 14, 311–321. doi: 10.1038/nrn3484

Cohen, P. Y., Breuer, R., and Wallach-Dayan, S. B. (2009). Thy1 up-regulates FasL expression in lung myofibroblasts via Src family kinases. *Am. J. Respir. Cell Mol. Biol.* 40, 231–238. doi: 10.1165/rcmb.2007-0348OC

Cohen, P. Y., Breuer, R., and Wallach-Dayan, S. B. (2018). A profibrotic phenotype in naive and in fibrotic lung myofibroblasts is governed by modulations in Thy-1 expression and activation. *Mediat. Inflamm.* 2018:4638437. doi: 10.1155/2018/4638437

Collins, M. D., and Keller, S. L. (2008). Tuning lipid mixtures to induce or suppress domain formation across leaflets of unsupported asymmetric bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 105, 124–128. doi: 10.1073/pnas.0702970105

Conference, B. (1997). Mutant mice and neuroscience: recommendations concerning genetic background. Banbury Conference on genetic background in mice. *Neuron* 19, 755–759. doi: 10.1016/S0896-6273(00)80958-7

Cullis, P. R., and Verkleij, A. J. (1979). Modulation of membrane structure by Ca2+ and dibucaine as detected by 31P NMR. *Biochim. Biophys. Acta* 552, 546–551. doi: 10.1016/0005-2736(79)90200-1

Errington, M. L., Bliss, T. V. P., Morris, R. J., Laroche, S., and Davis, S. (1997). Long-term potentiation in awake mutant mice. *Nature* 387, 666–667. doi: 10.1038/42625

Fiore, V. F., Ju, L., Chen, Y., Zhu, C., and Barker, T. H. (2014). Dynamic catch of a Thy-1-alpha5beta1+syndecan-4 trimolecular complex. *Nat. Commun.* 5:4886. doi: 10.1038/ncomms5886

Fiore, V. F., Strane, P. W., Bryksin, A. V., White, E. S., Hagood, J. S., and Barker, T. H. (2015). Conformational coupling of integrin and Thy-1 regulates Fyn priming and fibroblast mechanotransduction. *J. Cell Biol.* 211, 173–190. doi: 10.1083/jcb.201505007

Ford, M. J., Burton, L. J., Li, H., Graham, C. H., Frobert, Y., Grassi, J., et al. (2002). A marked disparity between the expression of prion protein and its message by neurones of the CNS. *Neuroscience* 111, 533–551. doi: 10.1016/S0306-4522(01)00603-0

Fu, B., and Vendruscolo, M. (2015). Structure and dynamics of intrinsically disordered proteins. *Adv. Exp. Med. Biol.* 870, 35–48. doi: 10.1007/978-3-319-20164-1_2

Fujita, M., and Kinoshita, T. (2012). GPI-anchor remodeling: potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. *Biochim. Biophys. Acta* 1821, 1050–1058. doi: 10.1016/j.bbalip.2012.01.004

Garcia-Manyes, S., Oncins, G., and Sanz, F. (2005). Effect of ion-binding and chemical phospholipid structure on the nanomechanics of lipid bilayers studied by force spectroscopy. *Biophys. J.* 89, 1812–1826. doi: 10.1529/biophysj.105.064030

Garcia-Manyes, S., Redondo-Morata, L., Oncins, G., and Sanz, F. (2010). Nanomechanics of lipid bilayers: heads or tails? *J. Am. Chem. Soc.* 132, 12874–12886. doi: 10.1021/ja1002185

Garcia-Parajo, M. F., Cambi, A., Torreno-Pina, J. A., Thompson, N., and Jacobson, K. (2014). Nanoclustering as a dominant feature of plasma membrane organization. *J. Cell Sci.* 127, 4995–5005. doi: 10.1242/jcs.146340

Griffiths, R. W., and Gleich, G. J. (1972). Proteolytic degradation of IgD and its relation to molecular conformation. *J. Biol. Chem.* 247, 4543–4548.

Han, X., Smith, N. L., Sil, D., Holowka, D. A., McLafferty, F. W., and Baird, B. A. (2009). IgE receptor-mediated alteration of membrane-cytoskeleton

interactions revealed by mass spectrometric analysis of detergent-resistant membranes. *Biochemistry* 48, 6540–6550. doi: 10.1021/bi900181w

Harding, A. S., and Hancock, J. F. (2008). Using plasma membrane nanoclusters to build better signaling circuits. *Trends Cell Biol.* 18, 364–371. doi: 10.1016/j.tcb.2008.05.006

He, H. T., Naquet, P., Caillol, D., and Pierres, M. (1991). Thy-1 supports adhesion of mouse thymocytes to thymic epithelial cells through a Ca2(+)-independent mechanism. *J. Exp. Med.* 173, 515–518. doi: 10.1084/jem.173.2.515

Heerklotz, H. (2002). Triton promotes domain formation in lipid raft mixtures. *Biophys. J.* 83, 2693–2701. doi: 10.1016/S0006-3495(02)75278-8

Heerklotz, H., Szadkowska, H., Anderson, T., and Seelig, J. (2003). The sensitivity of lipid domains to small perturbations demonstrated by the effect of Triton. *J. Mol. Biol.* 329, 793–799. doi: 10.1016/S0022-2836(03)00504-7

Hermosilla, T., Munoz, D., Herrera-Molina, R., Valdivia, A., Munoz, N., Nham, S. U., et al. (2008). Direct Thy-1/alphaVbeta3 integrin interaction mediates neuron to astrocyte communication. *Biochim. Biophys. Acta* 1783, 1111–1120. doi: 10.1016/j.bbamcr.2008.01.034

Herrera-Molina, R., Frischknecht, R., Maldonado, H., Seidenbecher, C. I., and Gundelfinger. (2012). Astrocytic alphaVbeta3 integrin inhibits neurite outgrowth and promotes retraction of neuronal processes by clustering Thy-1. *PLoS One* 7:e34295. doi: 10.1371/journal.pone.0034295

Herrera-Molina, R., Valdivia, A., Kong, M., Alvarez, A., Cardenas, A., Quest, A. F., et al. (2013). Thy-1-interacting molecules and cellular signaling in cis and trans. *Int. Rev. Cell Mol. Biol.* 305, 163–216. doi: 10.1016/B978-0-12-407695-2.00004-4

Hollrigel, G. S., Morris, R. J., and Soltensz, I. (1998). Enhanced inhibitory charge transfer during bursts of IPSCs in dentate granule cells in mice with regionally inhibited LTP. *Proc. R. Soc. Lond. B* 265, 63–69. doi: 10.1098/rspb.1998.0265

Honerkamp-Smith, A. R., Cicuta, P., Collins, M. D., Veatch, S. L., den Nijs, M., Schick, M., et al. (2008). Line tensions, correlation lengths, and critical exponents in lipid membranes near critical points. *Biophys. J.* 95, 236–246. doi: 10.1529/biophysj.107.128421

Horejsi, V., Zhang, W., and Schraven, B. (2004). Transmembrane adaptor proteins: organizers of immunoreceptor signalling. *Nat. Rev. Immunol.* 4, 603–616. doi: 10.1038/nri1414

Hueber, A. O., Bernard, A. M., Battari, C. L., Marguet, D., Massol, P., Foa, C., et al. (1997). Thymocytes in Thy-1-/- mice show augmented TCR signaling and impaired differentiation. *Curr. Biol.* 7, 705–708. doi: 10.1016/S0960-9822(06)00300-9

Hueber, A. O., Pierres, M., and He, H. T. (1992). Sulfated glycans directly interact with mouse Thy-1 and negatively regulate Thy-1-mediated adhesion of thymocytes to thymic epithelial cells. *J. Immunol.* 148, 3692–3699.

Huttner, W. B., and Zimmerberg, J. (2001). Implications of lipid microdomains for membrane curvature, budding and fission. *Curr. Opin. Cell Biol.* 13, 478–484. doi: 10.1016/S0955-0674(00)00239-8

Ipsen, J. H., Karlstrom, G., Mouritsen, O. G., Wennerstrom, H., and Zuckermann, M. J. (1987). Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta* 905, 162–172. doi: 10.1016/0005-2736(87)90020-4

Kiessling, V., Wan, C., and Tamm, L. K. (2009). Domain coupling in asymmetric lipid bilayers. *Biochim. Biophys. Acta* 1788, 64–71. doi: 10.1016/j.bbamem.2008.09.003

Kong, M., Munoz, N., Valdivia, A., Alvarez, A., Herrera-Molina, R., et al. (2013). Thy-1-mediated cell-cell contact induces astrocyte migration through the engagement of alphaVbeta3 integrin and syndecan-4. *Biochim. Biophys. Acta* 1833, 1409–1420. doi: 10.1016/j.bbamcr.2013.02.013

Kumar, A., Bhanja, A., Bhattacharyya, J., and Jaganathan, B. G. (2016). Multiple roles of CD90 in cancer. *Tumour. Biol.* 37, 11611–11622. doi: 10.1007/s13277-016-5112-0

Kuzmin, P. I., Akimov, S. A., Chizmadzhev, Y. A., Zimmerberg, J., and Cohen, F. S. (2005). Line tension and interaction energies of membrane rafts calculated from lipid splay and tilt. *Biophys. J.* 88, 1120–1133. doi: 10.1529/biophysj.104.048223

Lagos-Cabre, R., Alvarez, A., Kong, M., Burgos-Bravo, F., Cardenas, A., Rojas-Mancilla, E., et al. (2017). alphaVbeta3 Integrin regulates astrocyte reactivity. *J. Neuroinflamm.* 14:194. doi: 10.1186/s12974-017-0968-5

Lee, I. H., Saha, S., Polley, A., Huang, H., Mayor, S., Rao, M., et al. (2015). Live cell plasma membranes do not exhibit a miscibility phase transition over a wide range of temperatures. *J. Phys. Chem. B* 119, 4450–4459. doi: 10.1021/jp512839q

Leyton, L., Schneider, P., Labra, C. V., Ruegg, C., Hetz, C. A., Quest, A. F., et al. (2001). Thy-1 binds to integrin beta(3) on astrocytes and triggers formation of focal contact sites. *Curr. Biol.* 11, 1028–1038. doi: 10.1016/S0960-9822(01)00262-7

Lichtenberg, D., Ahyayauch, H., and Goni, F. M. (2013). The mechanism of detergent solubilization of lipid bilayers. *Biophys. J.* 105, 289–299. doi: 10.1016/j.bpj.2013.06.007

Lichtenberg, D., Goni, F. M., and Heerklotz, H. (2005). Detergent-resistant membranes should not be identified with membrane rafts. *Trends Biochem. Sci.* 30, 430–436. doi: 10.1016/j.tibs.2005.06.004

Lin, T. J., Lu, K. W., Chen, W. H., Cheng, C. M., and Lin, Y. W. (2015). Roles of syndecan-4 and relative kinases in dorsal root ganglion neuron adhesion and mechanotransduction. *Neurosci. Lett.* 592, 88–93. doi: 10.1016/j.neulet.2015.02.058

Liu, B., Chen, W., Evavold, B. D., and Zhu, C. (2014). Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* 157, 357–368. doi: 10.1016/j.cell.2014.02.053

Liu, X., Wong, S. S., Taype, C. A., Kim, J., Shentu, T. P., Espinoza, C. R., et al. (2017). Thy-1 interaction with Fas in lipid rafts regulates fibroblast apoptosis and lung injury resolution. *Lab. Invest.* 97, 256–267. doi: 10.1038/labinvest.2016.145

Madore, N., Smith, K. L., Graham, C. H., Jen, A., Brady, K., Hall, S., et al. (1999). Functionally different GPI proteins are organised in different domains on the neuronal surface. *EMBO J.* 18, 6917–6926. doi: 10.1093/emboj/18.24.6917

Maldonado, H., Calderon, C., Burgos-Bravo, F., Kobler, O., Zuschratter, W., Ramirez, O., et al. (2017). Astrocyte-to-neuron communication through integrin-engaged Thy-1/CBP/Csk/Src complex triggers neurite retraction via the RhoA/ROCK pathway. *Biochim. Biophys. Acta* 1864, 243–254. doi: 10.1016/j.bbamcr.2016.11.006

Mason, D. W., and Williams, A. F. (1980). The kinetics and binding to membrane antigens in solution and at the cell surface. *Biochem. J.* 187, 1–20. doi: 10.1042/bj1870001

Mayeux-Portas, V., File, S. E., Stewart, C. L., and Morris, R. J. (2000). Mice lacking the cell adhesion molecule Thy-1 fail to use socially-transmitted cues to direct their choice of food. *Curr. Biol.* 10, 68–75. doi: 10.1016/S0960-9822(99)00278-X

Mayor, S., Rothberg, K. G., and Maxfield, F. R. (1994). Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science* 264, 1948–1951. doi: 10.1126/science.7516582

Morris, R. (1992). Thy-1, the enigmatic extrovert on the neuronal surface. *BioEssays* 14, 715–722. doi: 10.1002/bies.950141014

Morris, R., Cox, H., Mombelli, E., and Quinn, P. J. (2004). Rafts, little caves and large potholes: how lipid structure interacts with membrane proteins to create functionally diverse membrane environments. *Subcell Biochem.* 37, 35–118. doi: 10.1007/978-1-4757-5806-1_2

Morris, R. J. (1994). "Antigen-antibody interactions: how affinity and kinetics affect assay design and selection procedures," in *Monoclonal Antibodies*, eds M. A. Ritter and H. Ladyman (Cambridge: Cambridge University Press), 34–59.

Morris, R. J. (2010). Ionic control of the metastable inner leaflet of the plasma membrane: fusions natural and artefactual. *FEBS Lett.* 584, 1665–1669. doi: 10.1016/j.febslet.2009.11.017

Morris, R. J., and Barber, P. C. (1983). Fixation of Thy-1 in nervous tissue for immunohistochemistry: a quantitative assessment of the effect of different fixation conditions upon retention of antigenicity and the cross-linking of Thy-1. *J. Histochem. Cytochem.* 31, 263–274. doi: 10.1177/31.2.6131917

Morris, R. J., Jen, A., and Warley, A. (2011). Isolation of nano-meso scale detergent resistant membrane that has properties expected of lipid 'rafts'. *J. Neurochem.* 116, 671–677. doi: 10.1111/j.1471-4159.2010.07076.x

Morris, R. J., and Nosten-Bertrand, M. (1996). NOS and aggression. *Trends Neurosci.* 19, 277–278. doi: 10.1016/S0166-2236(96)20025-6

Munro, S. (2003). Lipid rafts: elusive or illusive? *Cell* 115, 377–388. doi: 10.1016/S0092-8674(03)00882-1

Nemoto, Y. L., Morris, R. J., Hijikata, H., Tsunoyama, T. A., Shibata, A. C. E., Kasai, R. S., et al. (2017). Dynamic meso-scale anchorage of GPI-anchored receptors in the plasma membrane: prion protein vs. Thy1. *Cell Biochem. Biophys.* 75, 399–412. doi: 10.1007/s12013-017-0808-3

Nosten-Bertrand, M., Errington, M. L., Murphy, K. P. S. J., Tokugawa, Y., Barboni, E., Kozlova, E., et al. (1996). Normal spatial learning despite regional inhibition of LTP in mice lacking Thy-1. *Nature* 379, 826–829. doi: 10.1038/379826a0

Parkyn, C. J., Vermeulen, E. G., Mootoosamy, R. C., Sunyach, C., Jacobsen, C., Oxvig, C., et al. (2008). LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein. *J. Cell Sci.* 121, 773–783. doi: 10.1242/jcs.021816

Paulick, M. G., and Bertozzi, C. R. (2008). The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. *Biochemistry* 47, 6991–7000. doi: 10.1021/bi8006324

Picke, A. K., Campbell, G. M., Bluher, M., Krugel, U., Schmidt, F. N., Tsourdi, E., et al. (2018). Thy-1 (CD90) promotes bone formation and protects against obesity. *Sci. Transl. Med.* 10:eaao6806.

Puig, B., Altmeppen, H., and Glatzel, M. (2014). The GPI-anchoring of PrP: implications in sorting and pathogenesis. *Prion* 8, 11–18. doi: 10.4161/pri.27892

Raghupathy, R., Anilkumar, A. A., Polley, A., Singh, P. P., Yadav, M., Johnson, C., et al. (2015). Transbilayer lipid interactions mediate nanoclustering of lipid-anchored proteins. *Cell* 161, 581–594. doi: 10.1016/j.cell.2015.03.048

Rege, T. A., Pallero, M. A., Gomez, C., Grenett, H. E., Murphy-Ullrich, J. E., and Hagood, J. S. (2006). Thy-1, via its GPI anchor, modulates Src family kinase and focal adhesion kinase phosphorylation and subcellular localization, and fibroblast migration, in response to thrombospondin-1/hep I. *Exp. Cell Res.* 312, 3752–3767. doi: 10.1016/j.yexcr.2006.07.029

Shentu, T. P., Huang, T. S., Cernelc-Kohan, M., Chan, J., Wong, S. S., Espinoza, C. R., et al. (2017). Thy-1 dependent uptake of mesenchymal stem cell-derived extracellular vesicles blocks myofibroblastic differentiation. *Sci. Rep.* 7:18052. doi: 10.1038/s41598-017-18288-9

Shibata, A. C., Fujiwara, T. K., Chen, L., Suzuki, K. G., Ishikawa, Y., Nemoto, Y. L., et al. (2012). Archipelago architecture of the focal adhesion: membrane molecules freely enter and exit from the focal adhesion zone. *Cytoskeleton (Hoboken)* 69, 380–392. doi: 10.1002/cm.21032

Sibener, L. V., Fernandes, R. A., Kolawole, E. M., Carbone, C. B., Liu, F., McAffee, D., et al. (2018). Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell* 174, 672.e27–687.e27. doi: 10.1016/j.cell.2018.06.017

Storck, E. M., Ozbalci, C., and Eggert, U. S. (2018). Lipid cell biology: a focus on lipids in cell division. *Annu. Rev. Biochem.* 87, 839–869. doi: 10.1146/annurev-biochem-062917-012448

Sudhof, T. C. (2013). Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron* 80, 675–690. doi: 10.1016/j.neuron.2013.10.022

Sunyach, C., Jen, A., Deng, J., Fitzgerald, K., Frobert, Y., McCaffrey, M. W., et al. (2003). The mechanism of internalisation of GPI anchored prion protein. *EMBO J.* 22, 3591–3601. doi: 10.1093/emboj/cdg344

Surviladze, Z., Harrison, K. A., Murphy, R. C., and Wilson, B. S. (2007). FcepsilonRI and Thy-1 domains have unique protein and lipid compositions. *J. Lipid Res.* 48, 1325–1335. doi: 10.1194/jlr.M600485-JLR200

Suzuki, K. G., Fujiwara, T. K., Edidin, M., and Kusumi, A. (2007a). Dynamic recruitment of phospholipase C gamma at transiently immobilized GPI-anchored receptor clusters induces IP3-Ca2+ signaling: single-molecule tracking study 2. *J. Cell Biol.* 177, 731–742. doi: 10.1083/jcb.200609175

Suzuki, K. G., Fujiwara, T. K., Sanematsu, F., Iino, R., Edidin, M., and Kusumi, A. (2007b). GPI-anchored receptor clusters transiently recruit Lyn and G alpha for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J. Cell Biol.* 177, 717–730. doi: 10.1083/jcb.200609174

Suzuki, K. G., Kasai, R. S., Hirosawa, K. M., Nemoto, Y. L., Ishibashi, M., Miwa, Y., et al. (2012). Transient GPI-anchored protein homodimers are units for raft organization and function. *Nat. Chem. Biol.* 8, 774–783. doi: 10.1038/nchembio.1028

Suzuki, K. G. N., Ando, H., Komura, N., Konishi, M., Imamura, A., Ishida, H., et al. (2018). Revealing the raft domain organization in the plasma membrane by single-molecule imaging of fluorescent ganglioside analogs. *Methods Enzymol.* 598, 267–282. doi: 10.1016/bs.mie.2017.06.038

Tanimura, N., Saitoh, S., Kawano, S., Kosugi, A., and Miyake, K. (2006). Palmitoylation of LAT contributes to its subcellular localization and stability. *Biochem. Biophys. Res. Commun.* 341, 1177–1183. doi: 10.1016/j.bbrc.2006.01.076

Tian, T., Harding, A., Inder, K., Plowman, S., Parton, R. G., and Hancock, J. F. (2007). Plasma membrane nanoswitches generate high-fidelity Ras signal transduction. *Nat. Cell Biol.* 9, 905–914. doi: 10.1038/ncb1615

Tilcock, C. P., and Cullis, P. R. (1981). The polymorphic phase behaviour of mixed phosphatidylserine-phosphatidylethanolamine model systems as detected by 31P-NMR. *Biochim. Biophys. Acta* 641, 189–201. doi: 10.1016/0005-2736(81)90583-6

Tiveron, M. C., Barboni, E., Pliego Rivero, F. B., Gormley, A. M., Seeley, P. J., Grosveld, F., et al. (1992). Selective inhibition of neurite outgrowth on mature astrocytes by Thy-1 glycoprotein. *Nature* 355, 745–748. doi: 10.1038/355745a0

Tokutomi, S., Lew, R., and Ohnishi, S. (1981). Ca2+-induced phase separation in phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine mixed membranes. *Biochim. Biophys. Acta* 643, 276–282. doi: 10.1016/0005-2736(81)90073-0

Tomaselli, K. J., Doherty, P., Emmett, C. J., Damsky, C. H., Walsh, F. S., and Reichardt, L. F. (1993). Expression of beta 1 integrins in sensory neurons of the dorsal root ganglion and their functions in neurite outgrowth on two laminin isoforms. *J. Neurosci.* 13, 4880–4888. doi: 10.1523/JNEUROSCI.13-11-04880.1993

Tsunoyama, T. A., Watanabe, Y., Goto, J., Naito, K., Kasai, R. S., Suzuki, K. G. N., et al. (2018). Super-long single-molecule tracking reveals dynamic-anchorage-induced integrin function. *Nat. Chem. Biol.* 14, 497–506. doi: 10.1038/s41589-018-0032-5

Uversky, V. N. (2017). Intrinsically disordered proteins in overcrowded milieu: membrane-less organelles, phase separation, and intrinsic disorder. *Curr. Opin. Struct. Biol.* 44, 18–30. doi: 10.1016/j.sbi.2016.10.015

Valitutti, S., Muller, S., Cella, M., Padovan, E., and Tiveron, M. C. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 375, 148–151. doi: 10.1038/375148a0

van't Hof, W., and Resh, M. D. (2000). Targeting proteins to plasma membrane and membrane microdomains by N-terminal myristoylation and palmitoylation. *Methods Enzymol.* 327, 317–330. doi: 10.1016/S0076-6879(00)27287-X

Varisco, B. M., Ambalavanan, N., Whitsett, J. A., and Hagood, J. S. (2012). Thy-1 signals through PPARgamma to promote lipofibroblast differentiation in the developing lung. *Am. J. Respir. Cell Mol. Biol.* 46, 765–772. doi: 10.1165/rcmb.2011-0316OC

Veatch, S. L., Chiang, E. N., Sengupta, P., Holowka, D. A., and Baird, B. A. (2012). Quantitative nanoscale analysis of IgE-FcepsilonRI clustering and coupling to early signaling proteins. *J. Phys. Chem. B* 116, 6923–6935. doi: 10.1021/jp300197p

Wandel, E., Saalbach, A., Sittig, D., Gebhardt, C., and Aust, G. (2012). Thy-1 (CD90) is an interacting partner for CD97 on activated endothelial cells. *J. Immunol.* 188, 1442–1450. doi: 10.4049/jimmunol.1003944

Wetzel, A., Chavakis, T., Preissner, K. T., Sticherling, M., Haustein, U. F., Anderegg, U., et al. (2004). Human Thy-1 (CD90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J. Immunol.* 172, 3850–3859. doi: 10.4049/jimmunol.172.6.3850

Williams, A. F., Barclay, A. N., Letarte-Muirhead, M., and Morris, R. J. (1977). The tissue distribution, purification and chemical composition of the rat Thy-1 antigen. *Cold Spring Harbor. Symp. Quant. Biol.* 49, 51–61. doi: 10.1101/SQB.1977.041.01.009

Zhou, Y., Hagood, J. S., Lu, B., Merryman, W. D., and Murphy-Ullrich, J. E. (2010). Thy-1-integrin alphav beta5 interactions inhibit lung fibroblast contraction-induced latent transforming growth factor-beta1 activation and myofibroblast differentiation. *J. Biol. Chem.* 285, 22382–22393. doi: 10.1074/jbc.M110.126227

Zhou, Y., Hagood, J. S., and Murphy-Ullrich, J. E. (2004). Thy-1 expression regulates the ability of rat lung fibroblasts to activate transforming growth factor-beta in response to fibrogenic stimuli. *Am. J. Pathol.* 165, 659–669. doi: 10.1016/S0002-9440(10)63330-5

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Thy-1 (CD90) Signaling Preferentially Promotes RORγt Expression and a Th17 Response

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Thy-1 (CD90) is a glycosylphosphatidylinositol-anchored protein (GPI-AP) with signaling properties that is abundant on mouse T cells. Upon antibody-mediated crosslinking, Thy-1 provides a T cell receptor (TcR)-like signal that is sufficient to drive $CD4^+$ T cell proliferation and differentiation into effector cells when costimulatory signals are provided by syngeneic lipopolysaccharide-matured bone marrow-derived dendritic cells. In this study, we investigated the impact of Thy-1 signaling on the production of the T helper (Th) cell subset-associated cytokines, interferon (IFN) γ, interleukin (IL)-4 and IL-17A, as well as the *in vitro* polarization of highly purified resting $CD4^+$ T cells into Th1, Th2, and Th17 cells. Although $CD8^+$ T cells expressed more Thy-1 than $CD4^+$ T cells, both T cell populations were equally responsive to Thy-1 stimulation. In contrast to TcR stimulation of $CD3^+$ T cells, which favored IFNγ and IL-4 production, Thy-1 signaling favored IL-17 synthesis, indicating a previously unidentified difference between the consequences of Thy-1 and TcR signal transduction. Moreover, Thy-1 signaling preferentially induced the Th17-associated transcription factor RORγt in $CD4^+$ T cells. As with TcR signaling, Thy-1 stimulation of $CD4^+$ T cells under the appropriate polarizing conditions resulted in Th1, Th2 or Th17 cell induction; however, Thy-1 stimulation induced nearly 7- and 2-fold more IL-4 and IL-17A, respectively, but only slightly more IFNγ. The ability to provide a TcR-like signal capable of promoting T helper cell differentiation and cytokine synthesis was not common to all GPI-APs since cross-linking of Ly6A/E with mitogenic mAb did not promote substantial production of IFNγ, IL-4 or IL-17, although there was a substantial proliferative response. The preferential induction of RORγt and Th17 cytokine synthesis as a consequence of Thy-1 signaling suggests a default T helper cell response that may enhance host defense against extracellular pathogens.

Keywords: CD90, cytokine synthesis, glycosylphosphatidylinositol-anchored protein, T cell, Thy-1

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INTRODUCTION

Thy-1 is a 25 kDa glycosylphosphatidylinositol-anchored protein (GPI-AP) that is highly expressed on the surface of mouse thymocytes and peripheral T cells (Pont, 1987; Haeryfar and Hoskin, 2004). As with several other T cell-associated GPI-APs (Malek et al., 1994), cross-linking Thy-1 molecules with mitogenic anti-Thy-1 monoclonal antibody (mAb; clone G7) in the presence of costimulatory

syngeneic bone marrow-derived dendritic cells (BMDCs) results in T cell proliferation, interleukin (IL)-2 production and IL-2 receptor (CD25) expression (Haeryfar et al., 2003). The physiological ligand for T cell-associated Thy-1 has not yet been identified, although within the neurological compartment Thy-1 interacts with β_3 integrin on astrocytes to promote astrocyte adhesion (Leyton et al., 2001), while leukocyte-associated $\alpha_m\beta_2$ integrin promotes leukocyte adhesion to Thy-1-expressing endothelium (Wetzel et al., 2004). In addition, galactin-1, a soluble sugar binding protein, binds Thy-1 in a carbohydrate-dependent manner (Symons et al., 2000).

Since Thy-1 and other GPI-APs are localized within T cell lipid rafts, it is proposed that these GPI-APs induce T cell activation by the common mechanism of lipid raft aggregation and the subsequent activation of lipid raft-associated signaling molecules (Ilangumaran et al., 2000). Although T cell activation via Thy-1 crosslinking is at least partially dependent on expression of the complete T cell receptor (TcR) complex (Gunter et al., 1987), there are notable differences between Thy-1 and TcR stimulation (Furlong et al., 2017). Nevertheless, many of the same signaling molecules that are involved in TcR signaling have also been implicated in Thy-1 signaling. For example, Thy-1 signaling in T cells involves Ca^{+2} flux, activation of Lck, Fyn, and Zap-70 protein tyrosine kinases, mitogen-activated protein kinases (MAPKs), phospholipase C γ, protein kinase C, and phosphatidylinositol-3 kinase (Leyton et al., 1999; Haeryfar and Hoskin, 2001; Conrad et al., 2009; Furlong et al., 2017). Consistent with Thy-1 providing T cells with a TcR-like signal, Thy-1 stimulation in the presence of costimulatory BMDCs results in the development of fully armed cytotoxic T lymphocytes; however, Thy-1-induced cytotoxic T lymphocytes are deficient in granule-dependent cytotoxicity and function via the Fas/Fas ligand death receptor pathway (Kojima et al., 2000; Haeryfar et al., 2003; Furlong et al., 2017). Interestingly, Thy-1 crosslinking in the absence of costimulatory signaling causes $CD4^+$ T cells to express CD25 and exhibit regulatory function without expression of the T regulatory cell lineage-specific transcription factor FoxP3 (Conrad et al., 2012). However, the precise function of Thy-1 in T cell activation, differentiation and effector function is far from being completely understood.

Th1, Th2, and Th17 cells are $CD4^+$ T helper cell subsets with distinct cytokine profiles and biological functions (Fietta and Delsante, 2009). In the presence of interferon (IFN) γ and IL-12, naïve $CD4^+$ T cells differentiate into Th1 cells following antigen recognition (Lighvani et al., 2001; Mullen et al., 2001). Environments rich in IL-4 and IL-2 favor Th2 differentiation (Swain et al., 1990), whereas the development of Th17 cells is promoted by transforming growth factor (TGF)-β, IL-1β, IL-6 and/or IL-21 (Veldhoen et al., 2006; Acosta-Rodriguez et al., 2007; Korn et al., 2007). Development of each T helper cell subset is reciprocally regulated, ensuring efficient activation of only the most appropriate effector mechanisms (Fietta and Delsante, 2009). Th1 cells produce cytokines such as IFNγ that support cell-mediated immune responses against malignant cells, viruses and other intracellular pathogens, whereas Th2 cells are a source of IL-4 and other cytokines that support humoral immunity and allow for the elimination of extracellular pathogens, including helminthes. On the other hand, IL-17 and additional proinflammatory cytokines synthesized by Th17 cells are critical for protection against Gram-negative bacteria, fungi, and certain protozoal pathogens. The production of signature cytokines by Th1, Th2, and Th17 cells is regulated by lineage-specific transcription factors: T-bet for Th1 cytokines, GATA3 for Th2 cytokines, and RORγt for Th17 cytokines (Li et al., 2014). Dysregulation of T helper cell subsets can result in the pathogenesis of immune-mediated inflammatory diseases (Hirahara and Nakayama, 2016). A more thorough understanding of how T helper cell subsets develop will suggest new strategies for the treatment of infectious diseases, autoimmune and allergic disorders, and cancer.

In this study, we explored the effect of Thy-1 signaling, in the context of costimulatory signals from BMDCs, on T cell synthesis of cytokines that are typically associated with Th1 (IFNγ), Th2 (IL-4), and Th17 (IL-17A) $CD4^+$ T cell responses. TcR-activated T cells were used for comparison. We also compared expression of the lineage-specific transcription factors T-bet, GATA3 and RORγt by Thy-1- and TcR-activated T cells. In addition, the capacity of Thy-1 stimulation, under different T helper cell polarizing conditions, to induce the differentiation of $CD4^+$ T cells into Th1, Th2, and Th17 T helper cell subsets was also determined and compared to the effect of TcR stimulation under the same polarizing conditions. We show for the first time that T cell stimulation via Thy-1 in the absence of a polarizing environment preferentially induced a Th17 response. Under polarizing conditions, Thy-1 signaling, like TcR stimulation, promoted $CD4^+$ T cell differentiation into Th1, Th2, and Th17 subsets; however, Th1 responses were nearly equivalent whereas Th2 and Th17 responses were stronger in comparison to TcR-activated $CD4^+$ T cells.

MATERIALS AND METHODS

Animals

Adult female C57BL/6 mice, purchased at 6 to 8-weeks-of-age from Charles River Canada (Lasalle, QC, Canada), were housed in the Carleton Animal Care Facility at Dalhousie University and maintained on standard rodent chow and water supplied *ad libitium*. Animal protocols were approved by the Dalhousie University Committee on Laboratory Animals and were consistent with the Canadian Council on Animal Care Guidelines.

Medium

RPMI 1640 medium (Sigma-Aldrich, Oakville, ON, Canada) was supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamate, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4; Invitrogen; Burlington, ON, Canada). BMDC medium consisted of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin, 5 mM HEPES buffer and 50 μM β-mercaptoethanol (Sigma-Aldrich).

Cytokines and Antibodies

Recombinant mouse IFNγ, IL-12, IL-4, IL-6, and recombinant human TGF-β1 were purchased from Peprotech (Rocky Hill, NJ, United States). Recombinant mouse GM-CSF was from R&D Systems, Inc. (Minneapolis, MN, United States). Anti-Thy-1 mAb (clone G7, rat IgG2c), fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 mAb (clone 30-H12, rat IgG2b; clone 53-2.1, rat IgG2a) and rat IgG2c were purchased from BD Biosciences (Mississauga, ON, Canada). Anti-TcRβ mAb (clone H57-597, hamster IgG), anti-Ly6A/E mAb (clone D7, ratIgG2a), anti-IL-4 mAb (clone 11B11, rat IgG1), anti-IFNγ mAb (clone R4-6A2, rat IgG1), anti-IL-12/IL-23 p40 (clone C17.8, rat IgG2a), anti-RORγt mAb (clone B2D, rat IgG1κ), hamster IgG, phycoerythrin (PE)-conjugated anti-GATA3 (clone TWAJ, rat IgG2b), PE-conjugated anti-T-bet mAb (clone 4B10, mouse IgG1), FITC-conjugated anti-CD62L mAb (clone MEL-14, rat IgG2a), PE-conjugated antiCD44 mAb (clone IM7, rat IgG2b), FITC-conjugated rat IgG2b, FITC-conjugated rat IgG2a, FITC-conjugated hamster IgG, PE-conjugated rat IgG2b, PE-conjugated mouse IgG1, and PE-conjugated hamster IgG were purchased from eBioscience, Inc. (San Diego, CA, United States). FITC-conjugated anti-CD3ε mAb (clone 145-2C11, hamster IgG), and PE-conjugated anti-TcRαβ mAb (clone H57-597, hamster IgG) were purchased from Cedarlane Laboratories, Inc. (Hornby, ON, Canada). Anti-actin antibody (clone I-19), horse radish peroxidase (HRP)-conjugated anti-goat IgG, and HRP-conjugated anti-rat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

BMDC Preparation

BMDCs were prepared as described (Lutz et al., 1999). Briefly, tibias and femurs from euthanized mice were flushed with phosphate buffered saline (PBS) to create a single cell suspension. Erythrocytes were depleted by hypo-osmotic shock and the remaining bone marrow cells were resuspended in BMDC medium containing 20 ng/ml GM-CSF prior to being seeded into 6-well plates at 1×10^6 cells/well. After culture for 7 days at 37°C in a humidified 5% CO_2 incubator, non-adherent cells were treated with 1 μg/ml lipopolysaccharide (LPS, Sigma-Aldrich) for 24 h to promote dendritic cell maturation.

T Cell Isolation

Spleen cell or lymph node cell suspensions were prepared in ice-cold PBS using a tissue homogenizer. Erythrocytes were depleted by hypo-osmotic shock. Highly purified (>98%) $CD3^+$ T cells were obtained using the Pan T Cell Isolation MACS® kit from Miltenyi Biotec (Cambridge, MA, United States), as per the manufacturer's instructions. Highly purified $CD4^+$ or $CD8^+$ T cells were isolated by negative selection from lymph node cell preparations using $CD4^+$ or $CD8^+$ T Cell MACS® isolation kits, as per the manufacturer's instructions.

T Cell Activation

T cells in fully supplemented RPMI 1640 medium were plated in either 96-well U-bottom (2.5×10^5 cells/well) or 24-well flat-bottom plates ($1.25–2.5 \times 10^6$ cells/well) and activated with anti-TcRβ mAb, anti-Thy-1 mAb, anti-Ly6A/E in the presence of LPS-matured syngeneic BMDCs (8×10^3 cells/well or $4–8 \times 10^4$ cells/well, respectively) or 5 ng/ml phorbol 12-myristate 13-acetate (PMA), as indicated.

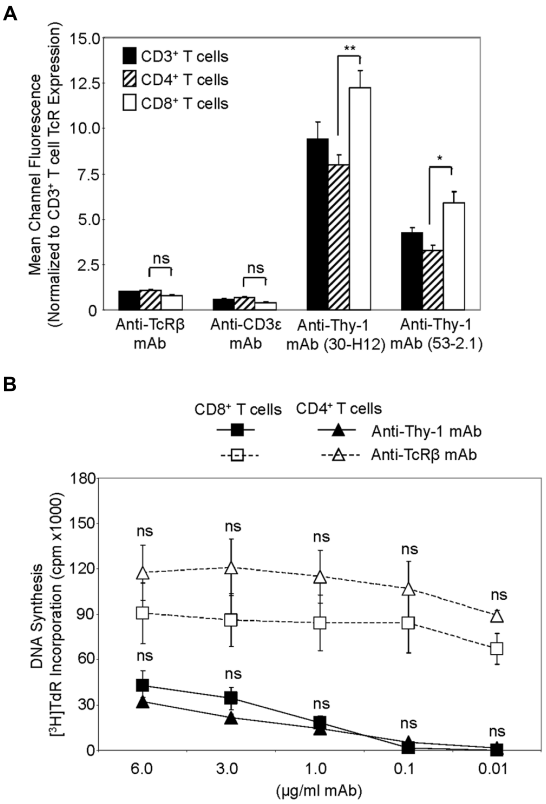

FIGURE 1 | Thy-1 versus TcR expression by $CD4^+$ and $CD8^+$ T cells, and relative response to Thy-1 and TcR signaling. **(A)** Highly purified $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells were stained with anti-TcRαβ-PE, anti-CD3ε-FITC, anti-Thy-1-FITC (clone 30-H12), anti-Thy-1-FITC (clone 53-2.1), or the appropriate FITC- or PE-labeled isotype control and analyzed by flow cytometry. Data are expressed as the average mean channel fluorescence normalized to TcR expression on $CD3^+$ T cells. Data are the mean ± SEM of three independent experiments; $^*p < 0.05$; $^{**}p < 0.001$; and ns, not-significant, as determined by ANOVA and the Bonferroni multiple comparisons post-test. **(B)** $CD4^+$ T cells or $CD8^+$ T cells with or without LPS-matured BMDCs, were seeded in triplicate into 96-well round-bottom plates, and then cultured in the presence of the indicated concentrations of anti-Thy-1 mAb (clone G7), anti-TcRβ mAb or isotype control for 72 h. Wells were pulsed with $[^3H]$TdR 6 h before the end of culture at which time the cells were harvested and DNA synthesis was determined based on $[^3H]$TdR incorporation. Background proliferation was controlled for by subtraction of experimental cpm from cpm of T cells and BMDC cultured alone (7288 ± 1488 for $CD8^+$ T cells and BMDCs, and 44157 ± 11919 for $CD4^+$ T cells and BMDCs) and are the mean ± SEM of three independent experiments; ns, not significant, as determined by ANOVA and the Bonferroni multiple comparisons post-test when the proliferation of $CD4^+$ T cells was compared to that of $CD8^+$ T cells that were activated by anti-Thy-1 or anti-TcRβ mAb.

$CD4^+$ T Cell Polarization

$CD4^+$ T cells were seeded into 24-well flat-bottom plates (1.25×10^6 cells/well) and activated with LPS-matured

syngeneic BMDCs (40×10^3 cells/well) and anti-TcRβ mAb or anti-Thy-1 mAb under different Th cell polarizing conditions: Th1 – 5 ng/ml IL-12 and 10 μg/ml anti-IL-4 mAb; Th2 – 10 ng/ml IL-4, 10 μg/ml anti-IL-12 mAb and 10 μg/ml anti-IFNγ mAb; Th17 – 100 ng/ml IL-6, 1 ng/ml TGFβ1, 10 μg/ml anti-IFNγ mAb, and 10 μg/ml anti-IL-4 mAb (Stritesky et al., 2008). On day 6, T cells were harvested and were restimulated with 5 ng/ml PMA plus 500 ng/ml ionomycin.

Tritiated-Thymidine Incorporation

Cultures performed in 96-well U-bottom plates were pulsed with 0.25 μCi of methyl ^{3}H-thymidine ([^{3}H]TdR; MP Biomedicals, Irvine, CA, United States) for 6 h. DNA was then harvested onto glass fiber filter mats using a Titer-Tek cell harvester (Skatron Instruments, Lier, Norway). [^{3}H]TdR incorporation, which is a measure of DNA synthesis, was determined using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter, Inc., Brea, CA, United States).

Flow Cytometry

For detection of cell-surface molecules, T cells were stained with FITC- or PE-conjugated mAbs or the appropriate isotype control, both at 10 μg/ml concentration. Briefly, T cells were resuspended in flow cytometry buffer (1% bovine serum albumin [w/v] and 0.2% sodium azide [w/v] in PBS), the desired mAb was added, and cells were incubated on ice in the dark for 45 min. Cells were then washed and fixed with 1% paraformaldehyde in PBS prior to flow cytometric analysis.

For intracellular staining of T-bet and GATA3, T cells were permeabilized and fixed using the FoxP3 staining kit (eBioscience), according to the manufacturer's instructions, and then stained with PE-conjugated anti-T-bet mAb (0.5 μg/ml), PE-conjugated anti-GATA3 mAb (0.06 μg/ml) or the appropriate isotype control for 45 min on ice in the dark. Cells were then washed and analyzed by flow cytometry.

For measurement of cell proliferation, T cells were labeled with 2.5 μM Oregon Green 488 dye (Invitrogen) in warm PBS for 10 min. Excess dye was inactivated by incubation for 30 min at 37°C in RPMI 1640 medium containing FCS. Cells were then washed and seeded at 2.5×10^6 cells/well into 24-well plates for activation as previously described. At the end of culture, cells were harvested and serial halving of fluorescence, which represents a round of cell division, was detected by flow cytometry.

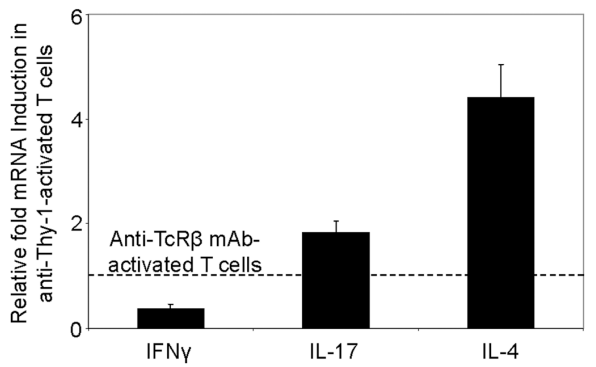

FIGURE 2 | Differential induction of T helper subset-associated cytokine mRNA by Thy-1 and TcR stimulation. Highly purified CD3$^+$ T cells with or without LPS-matured BMDCs were seeded into 24-well plates and then cultured in the presence or absence of 6 μg/ml anti-Thy-1 mAb (clone G7), anti-TcRβ mAb or appropriate isotype control for 24 h. Total RNA was isolated and used to generate cDNA. RT-PCR with primers specific for IFNγ, IL-17, IL-4 mRNA was performed. Pol II expression was used as a loading control. Relative expression of each cytokine mRNA was calculated using the standard curve method and normalized to the TcR-activated T cells. Data are the mean ± SEM of at least three separate experiments.

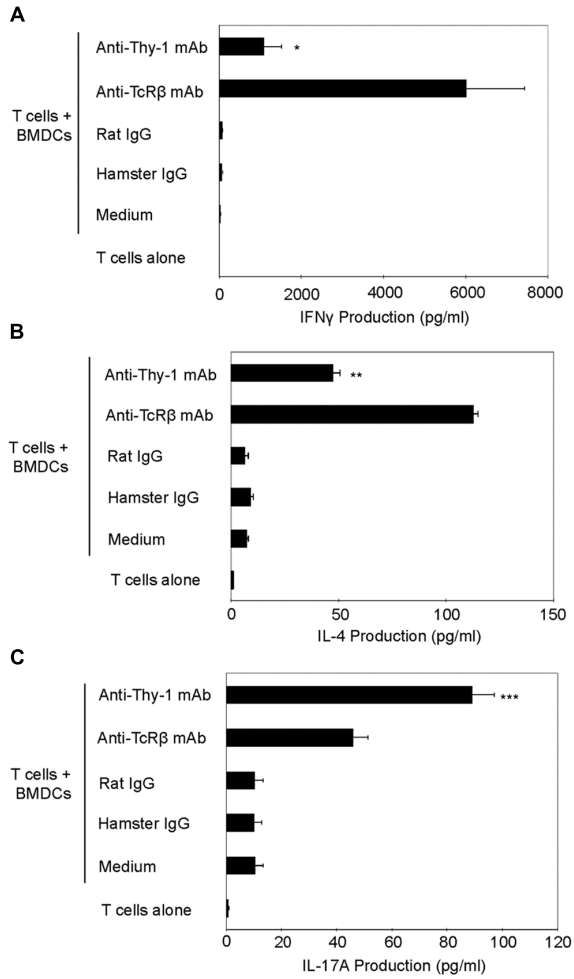

FIGURE 3 | Thy-1 signaling induces more IL-17A but less IL-4 and IFNγ synthesis by CD3$^+$ T cells in comparison to TcR signaling. **(A–C)** Highly purified CD3$^+$ T cells with or without LPS-matured BMDCs were seeded in quadruplicate into 96-well round-bottom plates and then cultured in the presence of 6 μg/ml anti-Thy-1 mAb (clone G7), anti-TcRβ mAb or the appropriate isotype control for the 24 h. Supernatants were isolated and analyzed by ELISA for **(A)** IFNγ **(B)** IL-4, and **(C)** IL-17A. Data shown are the mean of at least three separate experiments ± SEM; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ns, not significant, when compared to T cells activated with anti-TcRβ mAb and LPS-matured BMDCs, as determined by the Bonferroni multiple comparisons post-test.

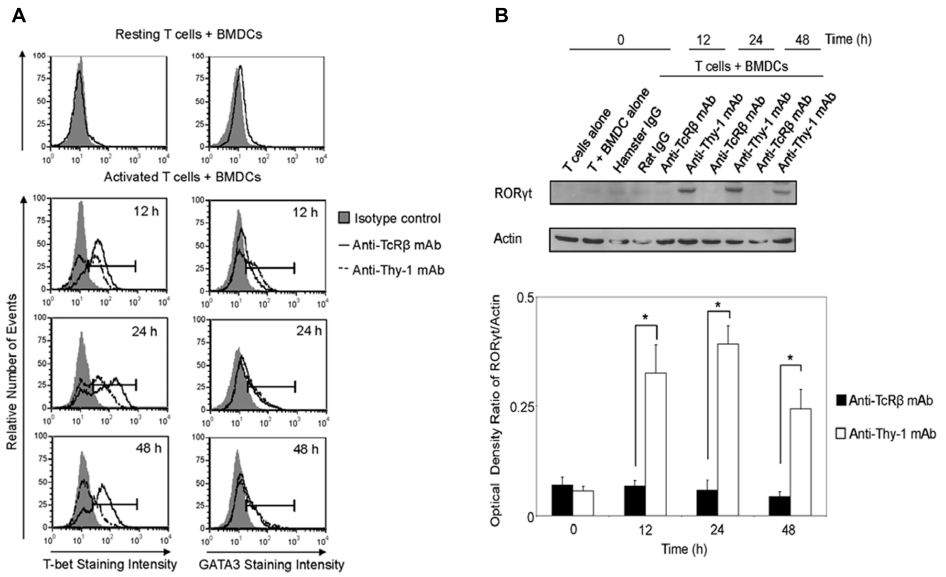

FIGURE 4 | Thy-1 signaling induces lower T-bet expression and higher GATA3 and RORγt expression in CD3$^+$ T cells in comparison to TcR signaling. **(A)** Highly purified CD3$^+$ T cells were seeded in quadruplicate into 96-well round-bottom plates with or without LPS-matured BMDCs, and were then cultured in the presence of 6 μg/ml anti-Thy-1 mAb (clone G7), anti-TcRβ mAb, or the appropriate isotype control for the indicated times. Cells were then fixed, permeabilized, and stained with PE-labeled anti-T-bet mAb, PE-labeled anti-GATA3 mAb, or the appropriate PE-labeled isotype control. Expression of T-bet and GATA3 was measured by flow cytometry. Data are representative of three separate experiments. **(B)** Highly purified CD3$^+$ T cells with or without LPS-matured BMDCs were seeded into 24-well plates and then cultured in the absence or presence of 6 μg/ml anti-Thy-1 mAb (clone G7), anti-TcRβ mAb, or the appropriate isotype control for the indicated times. Cell lysates were prepared and RORγt protein (58 kDa) levels were assessed by western blotting. Blots were reprobed for β-actin (42 kDa) to confirm equal loading of protein. Data are representative of three independent experiments. Optical density ratios were calculated by comparing the density of individual RORγt bands from three independent experiments with the corresponding β-actin bands. Data are shown as mean ± SEM; $*p < 0.001$, as determined by the Student's *t*-test.

Fluorescence intensity of individual cells was determined using a FACSCaliber flow cytometer with CellQuest software (version 3.3; Becton Dickinson; Mississauga, ON, United States). Data were analyzed using FCS Express software (verson 3.0; De Novo Software; Thornhill, ON, United States).

mRNA Expression

T cells were lysed in TRIzol reagent (Invitrogen) and total RNA was extracted as per the manufacturer's instructions. RNA was quantified using a spectrophotometer and RNA purity was determined based on the A260/A280 ratio. Moloney murine leukemia virus reverse transcriptase (Invitrogen) was used to reverse transcribe RNA, following the manufacturer's instructions. The resulting cDNA was then stored at −80°C for future use. Real time-polymerase chain reaction (RT-PCR) was carried out using the Quantifast SYBR-green RT-PCR kit (Qiagen; Mississauga, ON, United States). cDNA was amplified using the following primers: IFNγ, (F) 5′-ATG AAC GCT ACA CAC TGC ATC-3′, (R) 5′-CCA TCC TTT TGC CAG TTC CTC-3′; IL-4, (F) 5′-ACT TGA TGA GAG AGA TCA TCG GCA-3′, (R) 5′-AGC TCC ATG AGA ACA CTA GAG TT-3′; IL-17A, (F) 5′-CTC CAG AAG GCC CTC AGA CTA C-3′, (R) 5′-AGC TTT CCC TCC GCA TTA CAC AG-3′; and RNA polymerase II, (F) 5′-GCG GAT GAG GAT ATG CAA TAT GA-3′, (R) 5′-ACC AAG CCT TTC TCG TCA AAA TA-3′. RT-PCR reactions were performed using a MX3000P quantitative PCR machine (Stratagene; La Jolla, CA, United States). Cycling conditions were: 10 min activation step at 95°C, 40 amplification cycles at 95°C for 10 s, and 60°C for 30 s. Data were analyzed using Stratagene MxPro software, version 3.0. The size and integrity of RT-PCR products were verified using melt curve analysis and by running products on 3% agarose gels. Relative concentrations of mRNA were determined using the standard curve method, whereby standard curves are generated using serial dilutions of the cDNA from activated T cells. Cytokine mRNA levels were normalized to RNA polymerase II mRNA levels.

Enzyme-Linked Immunosorbant Assay (ELISA)

Cell-free supernatants from T cell cultures were assayed for IFNγ, IL-4 and IL-17A content using ELISA kits from eBioscience or BD Biosciences, according to the manufacturer's instructions. Absorbance at 450 nm with a wavelength correction for 570 nm was determined using an ELx800 UV universal microplate reader (Biotek Instruments, Inc., Winooski, VT, United States) and KCjunior software (version 1.17; Biotek Instruments, Inc.). SOFTmax® PRO software (version 4.3; Molecule Devices, Corp., Sunnyvale, CA, United States) was used to determine cytokine concentrations from the absorbance readings.

Western Blotting

Cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM Na_2HPO_4, 0.25% sodium deoxycholate [w/v], 0.1% Nonidet P-40 [v/v], 5 mM ethylenediaminetetraacetic

acid, and 5 mM ethyleneglycoltetraacetic acid) supplemented with fresh protease and phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 15–30 min on ice. Cellular debris was removed from the lysates by centrifugation at 10,000 *g*. Total protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, United States). Equal amounts of protein were added to sample buffer (200 mM Tris-HCl [pH 6.8], 30% glycerol [v/v], 6% sodium dodecyl sulfate [w/v], 15% β-mercaptoethanol [v/v], and 0.001% bromophenol [w/v]), which was then heated to 90–100°C for 5 min to promote protein denaturation. Lysates were stored at −80°C until use. Protein samples (10–20 μg protein/well) were loaded onto Tris-HCl acrylamide resolving gels and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate proteins, which were then transferred onto nitrocellulose membranes using an iBlot® Dry Blotting System (Invitrogen). Membranes were washed with Tris-buffered saline (TBS)-Tween-20 (TBST; 20 mM Tris-HCl [pH 7.6], 200 mM NaCl, 0.05% Tween-20 [v/v]) and blocked in TBST containing 5% fat-free milk powder [w/v] for 1 h at room temperature or overnight at 4°C with gentle rocking. Membranes were washed and then exposed to the primary antibody (1:200–1:1000 in TBST blocking solution) for 1 h at room temperature or overnight at 4°C with gentle rocking. Membranes were washed and then exposed to the appropriate HRP-conjugated secondary antibody (1:1000 in TBST blocking solution) for 1 h at room temperature with gentle rocking. Membranes were washed and reacted with enhanced chemiluminescence reagents (GE Healthcare, Baie d'Urfe, Quebec, CA, United States) for 1 min. Protein bands were visualized by exposure to X-ray film, which was developed in a Kodak X-OMAT 1000A automated X-ray developer.

Statistical Analysis

Data were analyzed using Instat software (GraphPad Software, Inc., San Diego, CA, United States). Student's *t*-test or one-way analysis of variance (ANOVA) with the Bonferroni multiple comparisons post-test were used as appropriate.

RESULTS

$CD4^+$ and $CD8^+$ T Cell Expression of, and Activation via, Thy-1

We first compared Thy-1 and TcR expression by $CD4^+$ and $CD8^+$ T cells, and their responsiveness to stimulation with anti-Thy-1 mAb versus anti-TcRβ mAb, in the presence of syngeneic BMDCs to provide costimulation. Flow cytometric analysis of highly purified $CD4^+$ and $CD8^+$ T cells, as well as unfractionated $CD3^+$ T cells, labeled with two different fluorescent anti-Thy-1 mAbs showed a significant difference in mean channel fluorescence, indicating that Thy-1 was more abundant on $CD8^+$ T cells than on $CD4^+$ T cells; in contrast, $CD8^+$ and $CD4^+$ T cell expression of TcRβ and CD3ε was similar (**Figure 1A**). Surprisingly, stimulation of highly purified $CD4^+$ and $CD8^+$ T cells with anti-Thy-1 mAb in the presence of BMDCs resulted in similar levels of DNA synthesis (**Figure 1B**), suggesting that differences in Thy-1 expression may not be functionally significant. DNA synthesis by $CD4^+$ and $CD8^+$ T cells in response to stimulation with anti-TcRβ mAb in the presence of BMDCs was substantially greater than the proliferative response to stimulation with anti-Thy-1 mAb under the same conditions, and at all mAb concentrations tested, which was in line with our earlier finding that Thy-1 cross-linking

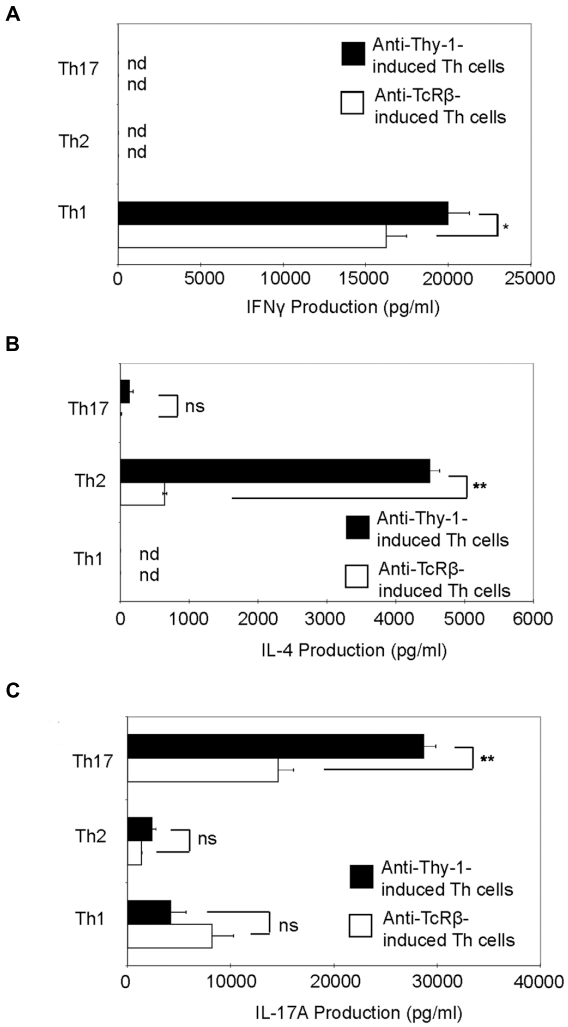

FIGURE 5 | Thy-1 signaling promotes Th1, Th2, and Th17 effector cell development in the presence of T helper cell subset-polarizing conditions. Highly purified $CD4^+$ T cells with or without LPS-matured BMDCs were seeded into 24-well flat-bottom plates and then stimulated with 6 μg/ml anti-Thy-1 mAb (clone G7) or anti-TcRβ mAb under Th1 (IL-12, anti-IL-4 mAb), Th2 (IL-4, anti-IL-12 mAb, anti-IFNγ mAb) or Th17 (IL-6, TGF-β, anti-IFNγ mAb, and anti-IL-4 mAb) polarizing conditions for 6 days. Polarized Th cells were washed, rested for 6 h and an equal number of viable cells were replated in 24-well flat-bottom plates, followed by restimulation with 5 ng/ml PMA and 500 ng/ml ionomycin and culture for 24 h. Supernatants were isolated and analyzed by ELISA for **(A)** IFNγ, **(B)** IL-4, and **(C)** IL-17A production. Data shown as mean ± SD are representative of three independent experiments; $*p = 0.02$; $**p < 0.001$; and ns, not significant, as determined by Student's *t*-test.

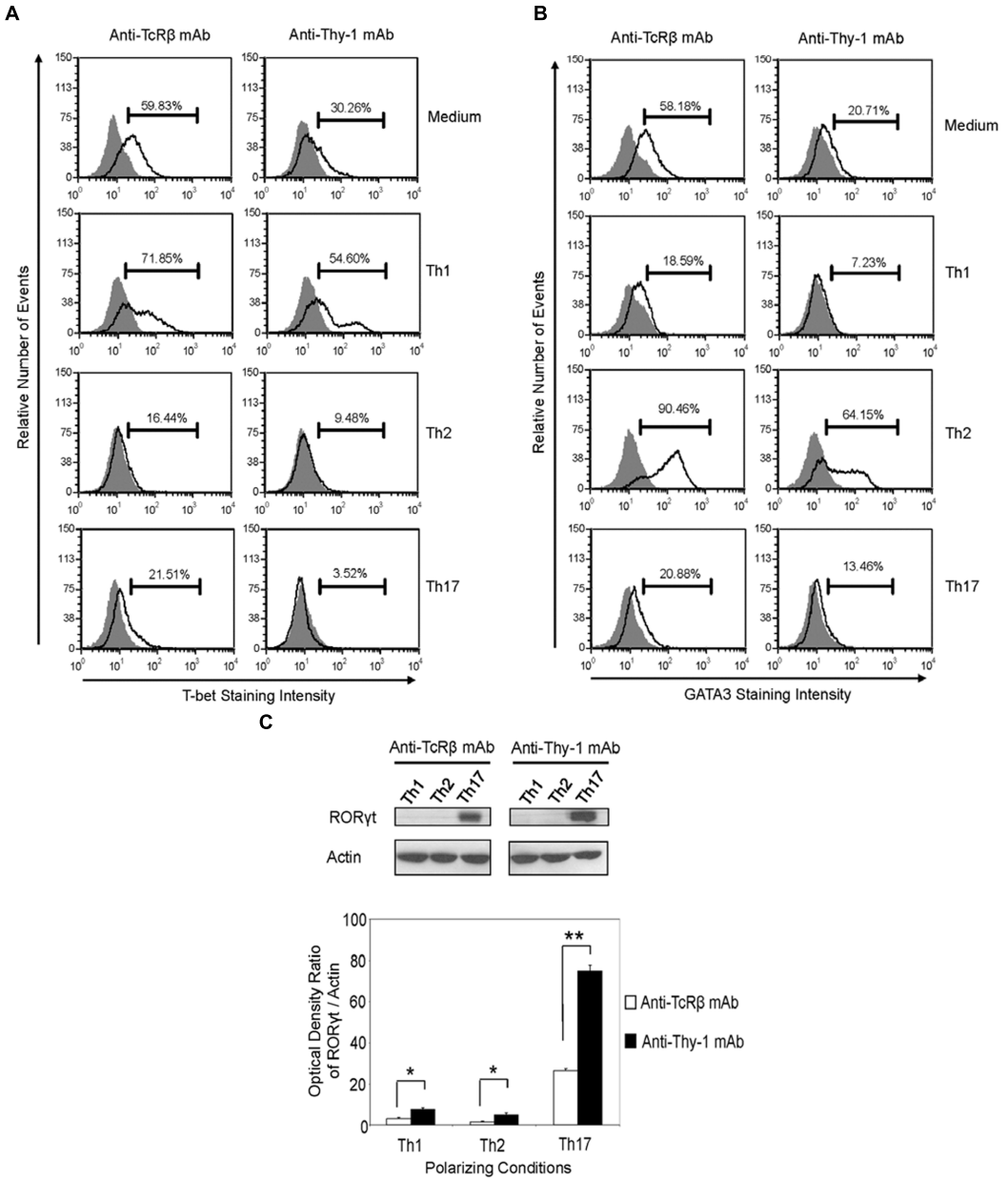

FIGURE 6 | Thy-1-induced Th1 and Th2 cells express less T-bet and GATA3, respectively, whereas Thy-1-induced Th17 cells express more RORγt than TcR-induced counterparts. Highly purified $CD4^+$ T cells with or without LPS-matured BMDCs were seeded into 24-well flat-bottom plates, and then stimulated with 6 μg/ml anti-Thy-1 mAb (clone G7) or anti-TcRβ mAb in the presence of medium alone or under Th1 (IL-12, anti-IL-4 mAb), Th2 (IL-4, anti-IL-12 mAb, anti-IFNγ mAb) or Th17 (IL-6, TGF-β, anti-IFNγ mAb, and anti-IL-4 mAb) polarizing conditions for 6 days. Cells were then fixed, permeabilized and stained for intracellular **(A)** T-bet (open peak) or **(B)** GATA3 (open peak), and compared with the appropriate isotype control (closed peak). Expression of T-bet and GATA3 was measured by flow cytometry. Data are representative of three separate experiments. **(C)** Cell lysates were prepared and RORγt protein (58 kDa) levels were assessed by western blotting. Blots were reprobed for β-actin (42 kDa) to confirm equal protein loading. Data are representative of three independent experiments. Optical density ratios were calculated by comparing the density of individual RORγt bands from three independent experiments with the corresponding β-actin bands. Data shown are the mean ± SEM; $*p < 0.05$; $**p < 0.001$, as determined by the Student's *t*-test.

provides a weaker activating signal than TcR cross-linking (Furlong et al., 2017). Subsequent experiments used anti-Thy-1 mAb and anti-TcRβ mAb at 6 μg/ml since this concentration of mAb induced a level of T cell activation that was not statistically different from that obtained with half the amount of mAb.

Differential Cytokine Response of Thy-1-Stimulated T Cells

We next used RT-PCR to compare the effect of Thy-1 and TcR stimulation of $CD3^+$ T cells on cytokine mRNA expression associated with Th1 (IFNγ), Th2 (IL-4), and Th17 (IL-17) cells.

Flow cytometric analysis revealed that 58% of $CD3^+$ T cells were $CD44^{low-medium}CD62L^+$ (naïve phenotype) and 15% were $CD44^{high}CD62L^+$ (effector/memory phenotype). **Figure 2** shows that, in comparison to TcR-activated T cells, Thy-1-activated T cells expressed substantially less IFNγ mRNA at 24 h post-activation; in contrast, IL-4 and IL-17A mRNA expression by Thy-1-activated T cells was significantly greater than that of TcR-activated T cells. ELISA measurements showed that at 24 h post-activation, Thy-1-stimulated $CD3^+$ T cell cultures contained significantly less IFNγ (**Figure 3A**) and more IL-17A (**Figure 3C**) than TcR-stimulated $CD3^+$ T cell cultures. In contrast, high levels of IL-4 mRNA expressed by Thy-1 stimulated T cells relative to TcR-stimulated T cells did not correlate with IL-4 protein expression, which was greater in TcR-stimulated T cells relative to Thy-1-stimulated T cells (**Figure 3B**).

Differential Expression of the T Helper Cell Lineage-Specific Transcription Factors by Thy-1-Stimulated T Cells

We next determined whether differential cytokine synthesis by $CD3^+$ T cells in response to Thy1- and TcR-signaling was associated with differential expression of the lineage-specific transcription factors, T-bet (Th1), GATA3 (Th2), and RORγt (Th17). As shown in **Figure 4A**, flow cytometry revealed that T-bet expression increased at 12, 24, and 48 h post-stimulation of $CD3^+$ T cells with anti-Thy-1 or anti-TcRβ mAb; however, Thy-1-activated T cells expressed less T-bet than TcR-activated T cells. A similar increase in GATA-3 expression by $CD3^+$ T cells at 24 and 48 h was observed following Thy-1- and TcR-stimulation. Although not sustained, more GATA-3 was expressed by Thy-1-activated $CD3^+$ T cells in comparison to TcR-activated $CD3^+$ T cells at 12 h post-stimulation. Co-cultures of unstimulated $CD3^+$ T cells and BMDCs expressed neither T-bet nor GATA3. Western blot analysis (**Figure 4B**) showed that Thy-1-activated $CD3^+$ T cells exhibited a marked increase in RORγt expression at 12, 24, and 48 h post-stimulation, whereas TcR-activated $CD3^+$ T cells failed to up-regulate RORγt expression. Co-cultures of unstimulated $CD3^+$ T cells and BMDCs failed to express RORγt.

Thy-1 Signaling Promotes $CD4^+$ T Cell Differentiation Into T Helper Cell Subsets

Th1, Th2, and Th17 T cell development is governed by the cytokine environment in which antigen-dependent $CD4^+$ T cell activation occurs (Fietta and Delsante, 2009). To determine whether Thy-1 stimulation supports Th1, Th2, and Th17 T cell differentiation, highly purified $CD4^+$ T cells were activated with anti-Thy-1 mAb in the presence of BMDCs under Th1-polarizing (IL-12, anti-IL-4 blocking mAb), Th2-polarizing (IL-4, and blocking mAb against IFNγ, and IL-12) or Th17-polarizing (IL-6, TGF-β1 and blocking mAb against IL-4 and IFNγ) conditions for 6 days. Flow cytometric analysis revealed that 61% of $CD4^+$ T cells were $CD44^{low-medium}CD62L^+$ (naïve phenotype) and 17% were $CD44^{high}CD62L^+$ (effector/memory phenotype). For comparison, parallel cultures were stimulated with anti-TcRβ mAb instead of anti-Thy-1 mAb. After polarization, $CD4^+$ T cells were restimulated with PMA/ionomycin, and IFNγ, IL-4 and IL-17A content in culture supernatants was determined by ELISA. As shown in **Figure 5**, $CD4^+$ T cells that were activated with anti-Thy-1 mAb (or anti-TcRβ mAb) under Th1-, Th2-, and Th17-polarizing conditions expressed the signature cytokines IFNγ (**Figure 5A**), IL-4 (**Figure 5B**), and IL-17A (**Figure 5C**), respectively. This finding is consistent with Thy-1 providing a TcR-like signal during T cell activation. Interestingly, in comparison to TcR signaling, Thy-1-stimulated $CD4^+$ T cell cultures contained nearly sevenfold more IL-4 and twofold more IL-17A under Th2 and Th17 polarizing conditions, respectively, but only slightly more IFNγ when activated in a Th1 polarizing environment, indicating that Thy-1 signaling is a strong inducer of T helper cell subset differentiation under polarizing conditions, especially Th2 and Th17 responses.

Differential Expression of Lineage-Specific Transcription Factors by Th1, Th2, and Th17 Cells Induced by Thy-1 Versus TcR Stimulation

Differentiation of Thy-1-stimulated $CD4^+$ T cells into Th1, Th2, and Th17 T cell subsets under the appropriate T helper cell polarizing conditions implied expression of the corresponding lineage-specific transcription factors. Indeed, highly purified $CD4^+$ T cells that were activated with anti-Thy-1 mAb in the presence of BMDCs under Th1 polarizing conditions showed increased T-bet expression that was not evident when Th2 or Th17 polarizing conditions were used (**Figure 6A**). As expected, this was also the case with TcR-stimulated $CD4^+$ T cells. In addition, both Thy-1- and TcR-stimulated $CD4^+$ T cells expressed GATA3 when activated under Th2 polarizing conditions but not under Th1 or Th17 polarizing conditions (**Figure 6B**). In comparison to TcR-induced Th1 and Th2 cells, slightly less T-bet and GATA3 was expressed by Thy-1-induced Th1 and Th2 cells, respectively. In contrast, Th17 cells that were induced by Thy-1 signaling under Th17 polarizing conditions expressed significantly more RORγt than Th17 cells that developed in response to TcR stimulation (**Figure 6C**). Th1 and Th2 cells that were stimulated with anti-Thy-1 mAb also expressed RORγt, albeit at a much lower level than Th17 cells that arose because of TcR or Thy-1 stimulation under Th17 polarizing conditions.

Ly6A/E-Stimulated T Cells Proliferate but Fail to Produce Th Cell Subset-Specific Cytokines

It is currently unclear whether the ability to provide a TcR-like signal that induces T helper cell subset cytokine synthesis is common to all T cell-associated GPI-APs or is unique to Thy-1. Ly6A/E is a GPI-AP that is known to promote T cell proliferation when cross-linked with mitogenic anti-Ly6A/E

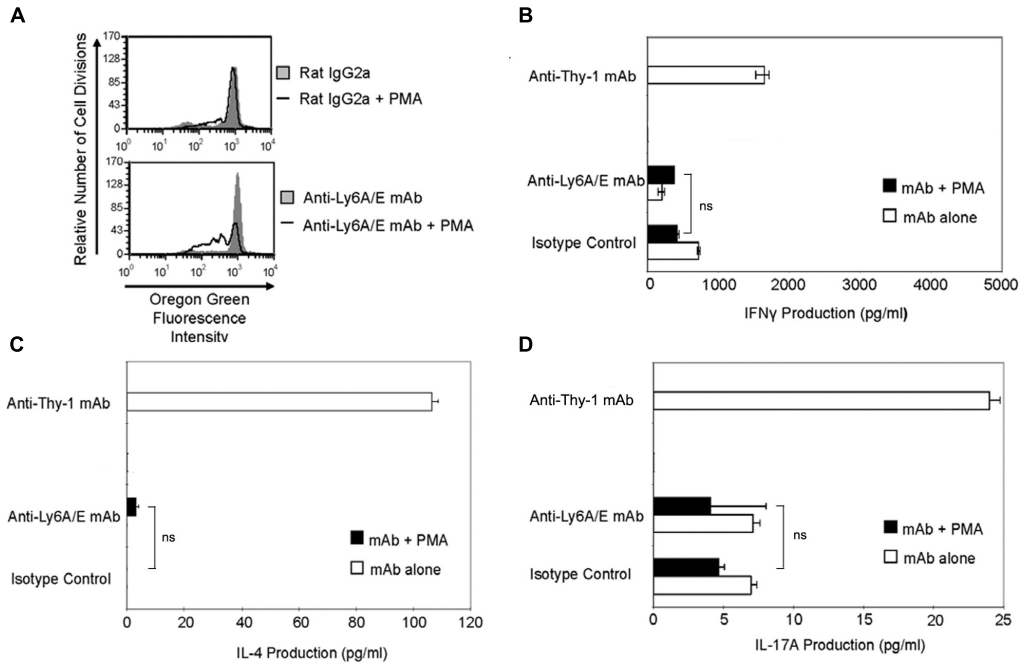

FIGURE 7 | Ly6A/E stimulation of T cells induces a weak proliferative response but minimal IFNγ, IL-4, and IL-17A synthesis. **(A)** Highly purified $CD3^+$ T cells were labeled with 2.5 μM Oregon Green 488 dye and seeded in duplicate into 96-well round-bottom plates. T cells were then cultured in the presence of 6 μg/ml anti-Ly6A/E mAb or the appropriate isotype control with or without 5 ng/ml PMA for the indicated times. Oregon Green 488 staining of pooled T cells was determined by flow cytometry. Fluorescence decreases with each round of cell division. Data shown are representative of three separate experiments. **(B)** $CD3^+$ T cells with or without LPS-matured BMDCs were seeded into 96-well round-bottom plates and then cultured in the presence of 6 μg/ml anti-Thy-1 mAb (clone G7) or anti-Ly-6A/E mAb with or without 5 ng/ml PMA, respectively, or the appropriate isotype control for 24 h. Supernatants were isolated and analyzed by ELISA for **(B)** IFNγ, **(C)** IL-4, and **(D)** IL-17A content. Data shown as mean ± SD of three technical replicates are representative of two independent experiments; ns, not significant by Student's *t*-test.

mAb in the presence of accessory cells and PMA (Malek et al., 1986). We therefore stimulated $CD3^+$ T cells with mitogenic anti-Ly6A/E mAb without or with PMA and measured cell proliferation by Oregon Green 488 staining, as well as IFNγ, IL-4, and IL-17A levels in culture supernatants by ELISA. PMA was used instead of BMDCs because we were unable to detect T cell proliferation following stimulation with anti-Ly6A/E mAb in the presence of BMDCs (data not shown), suggesting a deficiency in 1,2-diacylglycerol-dependent signaling following Ly6A/E crosslinking. As expected, $CD3^+$ T cells proliferated in the presence of anti-Ly6A/E mAb plus PMA (**Figure 7A**); however, in comparison to stimulation with anti-Thy-1 mAb in the presence of BMDCs, there was little or no production of IFNγ (**Figure 7B**), IL-4 (**Figure 7C**), and IL-17A (**Figure 7D**) when T cells were stimulated with anti-Ly6A/E mAb plus PMA. These data indicate that not all GPI-APs are able to signal for robust T helper cell cytokine synthesis.

DISCUSSION

The differentiation of $CD4^+$ T cells into phenotypically distinct T helper cell subsets in response to antigenic stimulation is crucial for an immune response that is appropriately tailored for optimal host defense (Fietta and Delsante, 2009). In this study, we provide evidence for the first time that the TcR-like signal induced by Thy-1 crosslinking resulted in the development of functional T helper cell subsets. Moreover, in comparison to TcR signaling, Thy-1 signaling in the absence of polarizing conditions preferentially promoted the synthesis of IL-17A over IFNγ and IL-4, which suggests a fundamental difference between signaling pathways associated with Thy-1 and the TcR. It is important to note that cytokine mRNA levels did not exactly correlate with protein expression. The failure of a gene's transcript level to predict its protein level is a well-known phenomenon that has been attributed to various factors, including translation efficiency and differences between mRNA and protein stability (Vogel et al., 2010; Schwanhäusser et al., 2011). Thy-1-stimulated T cells also showed lower expression of the Th1-defining transcription factor T-bet, which is expressed following TcR stimulation in the presence of IL-12 (Szabo et al., 2000). LPS-matured BMDCs secrete abundant IL-12 (Tada et al., 2004); however, in preliminary experiments we have observed that, in comparison to TcR-activated T cell cultures, significantly less IL-12 protein was present in Thy-1-stimulated T cell cultures (data not shown), which may account for reduced T-bet-dependent IFNγ expression. IL-4 synthesis by Thy-1-stimulated T cells was associated with an early and transient increase in expression of the Th2-defining transcription factor GATA3; at later time points Thy-1- and TcR-stimulated T cells

expressed equivalent amounts of GATA3. Thy-1 crosslinking generates a weaker T cell-activating signal than crosslinking of TcRs (Furlong et al., 2017), which may account for the transient increase in GATA3 expression since a weak TcR signal favors the development of Th2 cells (Pfeiffer et al., 1995). Strikingly, Thy-1 signaling preferentially induced expression of the Th17-defining transcription factor RORγt, which regulates IL-17 synthesis by binding directly to the *IL-17* promoter (Zhang et al., 2008). It is known that a strong TcR signal favors IL-17 production and Th17 differentiation in mouse T cells (Gomez-Rodriguez et al., 2009); hence, the difference between Thy-1 and TcR signal strength was not consistent with preferential RORγt expression and IL-17A synthesis by Thy-1-stimulated T cells. Rather, there appears to be a fundamental difference between Thy-1- and TcR-associated signaling pathways that regulate Th17 development. Since additional transcription factors such as Runx1 and STAT3 collaborate with RORγt to promote optimal *IL-17* gene transcription (Wei et al., 2007; Zhang et al., 2008), it will be important in future studies to determine whether Thy-1 signaling uniquely upregulates and/or activates any additional Th17-related transcription factors relative to TcR signaling.

In contrast to T cell-BMDC co-cultures, Thy-1 stimulation of $CD4^+$ T cells in the context of Th1, Th2, or Th17 polarizing cytokine environments clearly promoted Th1, Th2, and Th17 differentiation, respectively, indicating that Thy-1 provided an antigen-independent TcR-like signal that was sufficient to induce T helper cell subset differentiation. Moreover, Thy-1-activated $CD4^+$ T cells showed greater production of signature cytokines upon restimulation than did TcR-stimulated $CD4^+$ T cells, which suggests more efficient T helper cell polarization. Interestingly, IL-4 and IL-17A synthesis by Thy-1-stimulated $CD4^+$ T cells was markedly increased in comparison to TcR-stimulated $CD4^+$ T cells, suggesting that in the appropriate polarizing environment Thy-1 signaling preferentially promotes IL-4 and IL-17A synthesis. In contrast, IFNγ synthesis was only slightly increased following Thy-1 versus TcR stimulation of $CD4^+$ T cells. Surprisingly, in comparison to TcR stimulation, Thy-1 stimulated $CD4^+$ T cells expressed less T-bet and GATA3 under Th1 and Th2 polarizing conditions, respectively, suggesting that preferential activation of additional transcription factors involved in IFNγ and IL-4 gene expression (Kim et al., 1999; Samten et al., 2008) may contribute to increased synthesis of signature cytokines by Thy-1 stimulated $CD4^+$ T cells. On the other hand, enhanced IL-17A synthesis was correlated with increased RORγt expression by Thy-1-stimulated $CD4^+$ T cells.

The ability of Thy-1 to provide an antigen-independent TcR-like signal that promotes Th cell differentiation is not a common feature of all T cell-associated GPI-APs since mAb-mediated crosslinking of Ly6A/E in the presence of PMA failed to induce substantial IFNγ, IL-4, or IL-17A synthesis by T cells in comparison to Thy-1 stimulation. However, like Thy-1 signaling, Ly6A/E signaling resulted in T cell proliferation, suggesting at

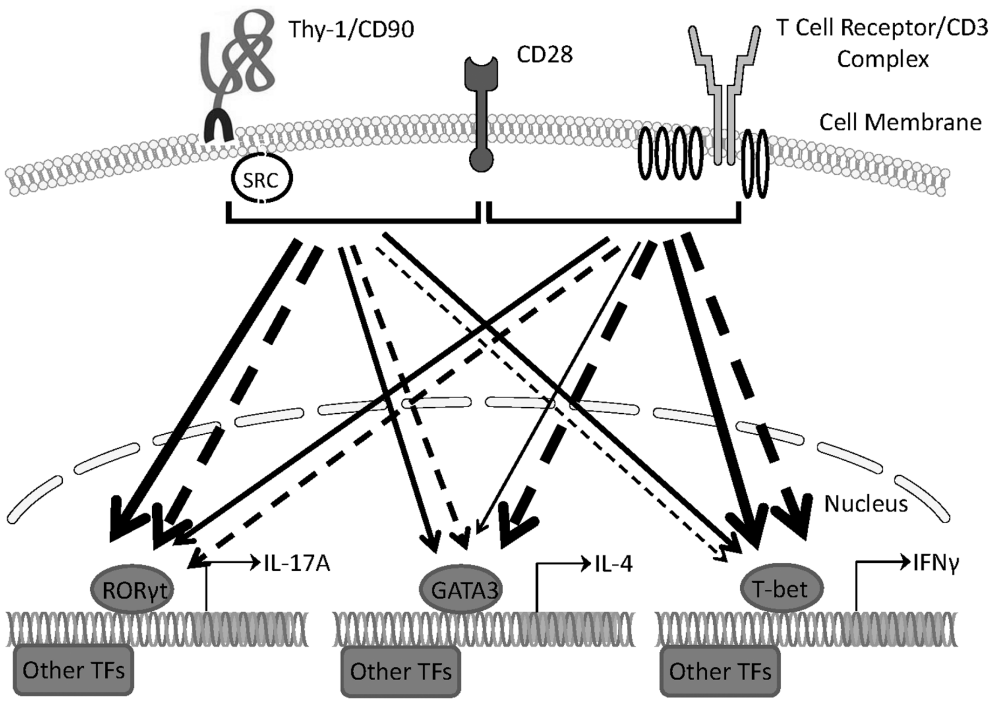

FIGURE 8 | Schematic comparing Th1, Th2, and Th17 responses induced by Thy-1 and TcR signaling in the absence or presence of polarizing environments. In the presence of a costimulatory signal from CD28, Thy-1 signaling in the absence of a T helper cell subset polarizing environment (dashed lines) favors a relatively strong Th17 response whereas TcR signaling favors Th1 and Th2 responses. In a T helper cell subset polarizing environment (solid lines), Thy-1 signaling is more effective than TcR signaling at inducing both Th2 and Th17 responses but induced a similar Th1 response. Thickness of lines indicates relative strength of the signal from Thy-1 versus TcR. TF denotes transcription factor.

least some induction of IL-2 needed to support T cell replication. Although signaling via other T cell-associated GPI-APs such as Ly6A/E may not support T helper cell differentiation, these cell-surface molecules may still affect T helper cell differentiation. For example, cellular prion protein has been implicated in the optimal production of T cell cytokines since T cells from cellular prion protein-deficient mice generate less IFNγ, IL-4, and IL-17 in response to TcR-stimulation and have altered responses to infection and autoantigens (Ingram et al., 2009).

CONCLUSION

Our findings provide evidence that Thy-1 signaling in the context of costimulation provided by BMDCs is sufficient to promote the differentiation of T helper cell effector subsets, albeit with different outcomes depending on whether or not a polarizing environment is present. **Figure 8** summarizes the effects of Thy-1 and TcR signaling in the absence or presence of Th1, Th2, or Th17 polarizing conditions. Our findings are consistent with a model in which Thy-1 crosslinking results in a weak TcR-like signal that preferentially promotes Th17 development in a non-polarizing environment, and Th2 and Th17 development under T helper cell subset polarizing conditions. We speculate that under physiological conditions, Thy-1 crosslinking by its physiological ligand, in combination with appropriate costimulatory signals, may result in enhanced antigen-independent host defense against extracellular pathogens.

AUTHOR CONTRIBUTIONS

SF and DH designed the study. SF and JG performed the experiments. SF, MC, JG, and DH analyzed the data and wrote the manuscript.

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REFERENCES

Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A., and Sallusto, F. (2007). Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol.* 8, 942–949. doi: 10.1038/ni1496

Conrad, D. M., Furlong, S. J., Doucette, C. D., Boudreau, R. T., and Hoskin, D. W. (2009). Role of mitogen-activated protein kinases in Thy-1-induced T-lymphocyte activation. *Cell. Signal.* 21, 1298–1307. doi: 10.1016/j.cellsig.2009.03.014

Conrad, D. M., Power Coombs, M. R., Furlong, S. F., Forward, N. R., and Hoskin, D. W. (2012). Induction of $CD4^+CD25^+FoxP3^-$ regulatory T cells by Thy-1 stimulation of $CD4^+$ T cells. *Immunol. Cell Biol.* 90, 248–252. doi: 10.1038/icb.2011.33

Fietta, P., and Delsante, G. (2009). The effector T helper cell triade. *Riv. Biol.* 102, 61–74.

Furlong, S. J., Power Coombs, M. R., and Hoskin, D. W. (2017). Thy-1 stimulation of mouse T cells induces a delayed T cell receptor-like signal that results in Ca^{2+}-independent cytotoxicity. *Mol. Med. Rep.* 16, 5683–5692. doi: 10.3892/mmr.2017.7242

Gomez-Rodriguez, J., Sahu, N., Handon, R., Davidson, T. S., Anderson, S. M., Kirby, M. R., et al. (2009). Differential expression of interleukin-17A and -17F is coupled to T cell receptor signaling via inducible T cell kinase. *Immunity* 31, 587–597. doi: 10.1016/j.immuni.2009.07.009

Gunter, K. C., Germain, R. N., Kroczek, R. A., Saito, T., Yokoyama, W. M., Chan, C., et al. (1987). Thy-1-mediated T-cell activation requires co-expression of CD3/Ti complex. *Nature* 326, 505–507. doi: 10.1038/326505a0

Haeryfar, S. M., Al-Alwan, M. M., Mader, J. S., Rowden, G., West, K. A., and Hoskin, D. W. (2003). Thy-1 signaling in the context of costimulation provided by dendritic cells provides signal 1 for T cell proliferation and cytotoxic effector molecule expression, but fails to trigger delivery of the lethal hit. *J. Immunol.* 171, 69–77. doi: 10.4049/jimmunol.171.1.69

Haeryfar, S. M., and Hoskin, D. W. (2001). Selective pharmacological inhibitors reveal differences between Thy-1- and T cell receptor-mediated signal transduction in mouse T lymphocytes. *Int. Immunopharmacol.* 1, 689–698. doi: 10.1016/S1567-5769(01)00002-9

Haeryfar, S. M., and Hoskin, D. W. (2004). Thy-1: more than a mouse pan-T cell marker. *J. Immunol.* 173, 3581–3588. doi: 10.4049/jimmunol.173.6.3581

Hirahara, K., and Nakayama, T. (2016). CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. *Int. Immunol.* 28, 163–171. doi: 10.1093/intimm/dxw006

Ilangumaran, S., He, H. T., and Hoessli, D. C. (2000). Microdomains in lymphocyte signalling: beyond GPI-anchored proteins. *Immunol. Today* 21, 2–7. doi: 10.1016/S0167-5699(99)01494-2

Ingram, R. J., Isaacs, J. D., Kaur, G., Lowther, D. E., Reynolds, C. J., Boyton, R. J., et al. (2009). A role of cellular prion protein in programming T-cell cytokine responses in disease. *FASEB J.* 23, 1672–1684. doi: 10.1096/fj.08-116087

Kim, J. I., Ho, I. C., Grusby, M. J., and Glimcher, L. H. (1999). The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity* 10, 45–51. doi: 10.1016/S1074-7613(00)80073-4

Kojima, H., Toda, M., and Sitkovsky, M. V. (2000). Comparison of Fas- versus perforin-mediated pathways of cytotoxicity in TCR- and Thy-1-activated murine T cells. *Int. Immunol.* 12, 365–374. doi: 10.1093/intimm/12.3.365

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T. B., et al. (2007). IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells. *Nature* 448, 484–487. doi: 10.1038/nature05970

Leyton, L., Quest, A. F., and Bron, C. (1999). Thy-1/CD3 coengagement promotes TCR signaling and enhances particularly tyrosine phosphorylation of the raft molecule LAT. *Mol. Immunol.* 36, 755–768. doi: 10.1016/S0161-5890(99)00086-3

Leyton, L., Schneider, P., Labra, C. V., Ruegg, C., Hetz, C. A., Quest, A. F., et al. (2001). Thy-1 binds to integrin β3 on astrocytes and triggers formation of focal contact sites. *Curr. Biol.* 11, 1028–1038. doi: 10.1016/S0960-9822(01)00262-7

Li, P., Spolski, R., Liao, W., and Leonard, W. J. (2014). Complex interactions of transcription factors in mediating cytokine biology in T cells. *Immunol. Rev.* 261, 141–156. doi: 10.1111/imr.12199

Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., et al. (2001). T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 15137–15142. doi: 10.1073/pnas.261570598

Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rössner, S., Koch, F., Romani, N., et al. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223, 77–92. doi: 10.1016/S0022-1759(98)00204-X

Malek, T. R., Fleming, T. J., and Codias, E. K. (1994). Regulation of T lymphocyte function by glycosylphosphatidylinositol (GPI)-anchored proteins. *Semin. Immunol.* 6, 105–113. doi: 10.1006/smim.1994.1015

Malek, T. R., Ortega, G., Chan, C., Kroczek, R. A., and Shevach, E. M. (1986). Role of Ly-6 in lymphocyte activation. II. Induction of T cell activation by monoclonal anti-Ly-6 antibodies. *J. Exp. Med.* 164, 709–722. doi: 10.1084/jem.164.3.709

Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., et al. (2001). Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292, 1907–1910. doi: 10.1126/science.1059835

Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A., and Bottomly, K. (1995). Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med.* 181, 1569–1574. doi: 10.1084/jem.181.4.1569

Pont, S. (1987). Thy-1: a lymphoid cell subset marker capable of delivering an activation signal to mouse T lymphocytes. *Biochimie* 69, 315–320. doi: 10.1016/0300-9084(87)90022-8

Samten, B., Townsend, J. C., Weis, S. E., Boumik, A., Klucar, P., Shams, H., et al. (2008). CREB, ATF, and AP-1 transcription factors regulate IFN-γ secretion by human T cells in response to mycobacterial antigen. *J. Immunol.* 181, 2056–2064. doi: 10.4049/jimmunol.181.3.2056

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., et al. (2011). Global quantification of mammalian gene expression control. *Nature* 473, 337–342. doi: 10.1038/nature10098

Stritesky, G. L., Yeh, N., and Kaplan, M. H. (2008). IL-23 promotes maintenance but not commitment to the Th17 lineage. *J. Immunol.* 181, 5948–5955. doi: 10.4049/jimmunol.181.9.5948

Swain, S. L., Weinberg, A. D., English, M., and Huston, G. (1990). IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145, 3796–3806.

Symons, A., Cooper, D. N., and Barclay, A. N. (2000). Characterization of the interaction between galectin-1 and lymphocyte glycoproteins CD45 and Thy-1. *Glycobiology* 10, 559–563. doi: 10.1093/glycob/10.6.559

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100, 655–669. doi: 10.1016/S0092-8674(00)80702-3

Tada, Y., Asahina, A., Fujita, H., Mitsui, H., Torii, H., Watanabe, T., et al. (2004). Differential effects of LPS and TGF-β on the production of IL-6 and IL-12 by Langerhans cells, splenic dendritic cells, and macrophages. *Cytokine* 25, 155–161. doi: 10.1016/j.cyto.2003.11.006

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. (2006). TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24, 179–189. doi: 10.1016/j.immuni.2006.01.001

Vogel, C., Abreu Rde, S., Ko, D., Shapiro, B. A., Burns, S. C., Sandu, D., et al. (2010). Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. *Mol. Syst. Biol.* 6:400. doi: 10.1038/msb.2010.59

Wei, L., Laurence, A., Elias, K. M., and O'Shea, J. J. (2007). IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J. Biol. Chem.* 282, 34605–34610. doi: 10.1074/jbc.M705100200

Wetzel, A., Chavakis, T., Preissner, K. T., Sticherling, M., Haustein, U. F., Anderegg, U., et al. (2004). Human Thy-1 (CD90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J. Immunol.* 172, 3850–3859. doi: 10.4049/jimmunol.172.6.3850

Zhang, F., Meng, G., and Strober, W. (2008). Interactions among the transcription factors Runx1, RORγt and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat. Immunol.* 9, 1297–1306. doi: 10.1038/ni.1663

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REVIEW
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Thy-1/CD90 a Bidirectional and Lateral Signaling Scaffold

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Thy-1/CD90 is a glycoprotein attached to the outer face of the plasma membrane with various functions, which depend on the context of specific physiological or pathological conditions. Many of these reported functions for Thy-1/CD90 arose from studies by our group, which identified the first ligand/receptor for Thy-1/CD90 as an integrin. This finding initiated studies directed toward unveiling the molecular mechanisms that operate downstream of Thy-1/CD90 activation, and its possible interaction with proteins in the membrane plane to regulate their function. The association of Thy-1/CD90 with a number of cell surface molecules allows the formation of extra/intracellular multiprotein complexes composed of various ligands and receptors, extracellular matrix proteins, intracellular signaling proteins, and the cytoskeleton. The complexes sense changes that occur inside and outside the cells, with Thy-1/CD90 at the core of this extracellular molecular platform. Molecular platforms are scaffold-containing microdomains where key proteins associate to prominently influence cellular processes and behavior. Each component, by itself, is less effective, but when together with various scaffold proteins to form a platform, the components become more specific and efficient to convey the messages. This review article discusses the experimental evidence that supports the role of Thy-1/CD90 as a membrane-associated platform (ThyMAP).

Keywords: GPI-anchor, PLATFORM, membrane-associated, integrin, syndecan 4, Thy-1 (CD90)

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INTRODUCTION

The glycosyl-phosphatidylinositol (GPI)-anchored protein Thy-1/CD90 is a resident of lipid rafts abundantly expressed in neurons, thymocytes, and some fibroblasts. Thy-1/CD90 is an integrin ligand or receptor that mediates cell-to-cell contacts that trigger changes in both cells involved. Despite being a plasma membrane-associated protein, Thy-1/CD90 holds some features of extracellular matrix (ECM) proteins. It possesses an integrin binding site (RGD-like tripeptide: RLD) and a heparin binding domain (HBD: REKRK). Through these sites,

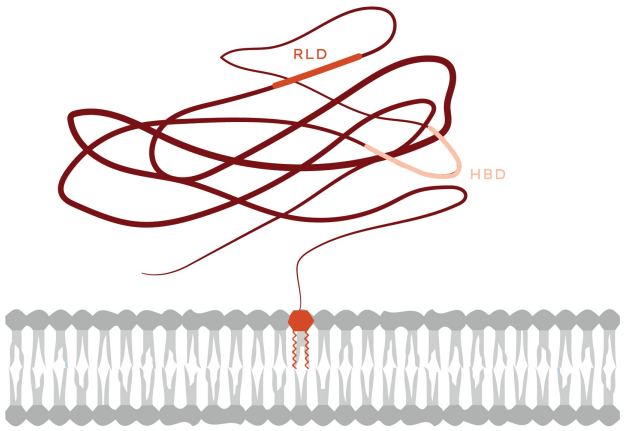

FIGURE 1 | Schematic representation of the Thy-1/CD90 protein. The Thy-1/CD90 GPI anchor appears inserted in the outer leaflet of the plasma membrane. The Thy-1/CD90 regions that interact with integrins (RLD) and Syndecan-4 (HBD, heparin-binding domain) are indicated.

Thy-1/CD90 binds to integrins and syndecan-4 (SDC4) receptors, respectively (**Figure 1**), andpromotes/regulates cellular contraction, adhesion and migration (Herrera-Molina et al., 2013; Leyton and Hagood, 2014).

Since its discovery in 1964, evidence has indicated that Thy-1/CD90 interacts with various proteins: different types of integrins, SDC4, Csk-binding protein (CBP), ion channels, and CD97 [reviewed in Herrera-Molina et al. (2013)]. These associations can occur between opposite cells (*in trans*) to trigger signal transduction pathways, or in the plane of the membrane (*in cis*) to regulate protein function and signaling. Along with these interactions come numerous functions, such as T cell activation, neuronal process retraction, cell adhesion and migration, fibroblast and dendritic cell differentiation [reviewed in Herrera-Molina et al. (2013)]. Other unanticipated functions have also been reported, such as those recently revealed in osteogenic differentiation of mesenchymal stem cells (Picke et al., 2018a,b) in mechanotransduction of lung fibroblasts (Fiore et al., 2015) and in tumor cell migration/invasion [reviewed in Herrera-Molina et al. (2013) and Sauzay et al. (2019)].

Thy-1/CD90 also exist as a soluble protein, although it has been detected at very low concentrations (ng/ml) in body fluids. The function of this form of Thy-1/CD90 is unknown, but it is speculated that it could serve as a competitor of the membranous form of the protein (Leyton and Hagood, 2014). Interestingly, Thy-1 has also been found forming part of extracellular vesicles (Hagood, 2019). Various authors in this Frontiers research topic have reviewed these forms and functions in detail (Furlong et al., 2018; Morris, 2018; Hagood, 2019; Hu and Barker, 2019; Sauzay et al., 2019). Therefore, we will focus here on those functions that are more relevant to Thy-1/CD90 signaling mechanisms, the formation of multiprotein complexes, and how Thy-1/CD90 in microdomains, as part of these complexes, confines its dynamic nature to regulate various cellular responses.

THY-1/CD90 CELL ADHESION MOLECULE, A RECEPTOR OR A LIGAND?

Our findings reported in 2001 describe that neuronal Thy-1/CD90 binds to $\alpha_v\beta_3$ integrin in astrocytes and that this interaction induces astrocyte adhesion to the ECM (Leyton et al., 2001; Hermosilla et al., 2008). Later, this interaction was reported to trigger signals both *in cis* and *in trans*, and to require the additional binding of Thy-1/CD90 to SDC4 receptor in order to promote astrocyte responses (Avalos et al., 2009; Herrera-Molina et al., 2012, 2013). In neurons, Thy-1/CD90 is in a preformed membrane complex that includes the transmembrane protein CBP and the non-receptor tyrosine kinase Src (**Figure 2A**); when engaged by an integrin, Thy-1/CD90 transduces a signal through the membrane complex with CBP, which recruits Csk, inactivates Src, and leads to the activation of the small G protein RhoA (**Figure 2B**). The activation of this signaling pathway leads to the contraction of neuronal processes (axons and dendrites) (Herrera-Molina et al., 2012; Maldonado et al., 2017). On the other hand, in astrocytes, integrin and SDC4 engagement by Thy-1/CD90 generates a response that activates the non-receptor tyrosine kinases FAK and Src, recruiting various proteins, including paxillin, vinculin, p130Cas, and forming the multimolecular adhesome complex (Avalos et al., 2009; Kong et al., 2013). In this case, the Rho GTPase cycle is also activated, thereby modulating the actin cytoskeleton and helping astrocytes to adhere and move (Avalos et al., 2004; Maldonado et al., 2017). In this cell-to-cell association, and because the interaction triggers responses in both cells in a bidirectional manner, the receptor could be either Thy-1/CD90 or $\alpha_v\beta_3$ integrin/SDC4, depending on the process or cell under study.

RhoA, Rac1, and Cdc42 are the most studied GTPases of the Rho family. When activated by the exchange of GDP for GTP, these proteins regulate effector molecules that control the actin cytoskeleton and thus, modulate cell polarization, adhesion, and migration. The activation of Rho GTPases is controlled by various proteins, including Guanine-nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs), and GDP dissociation inhibitors (GDIs). RhoGTPases are anchored to the membrane through their prenylated tail and are kept in the cytosol by GDI proteins, which sequester the tail of Rho proteins (Burridge and Wennerberg, 2004; Boulter et al., 2010). Astrocytes stimulated with Thy-1/CD90 increase RhoA activity in the first 20 min, whereas Rac1 activity decreases and starts to rise after 30 min of stimulation (Kong et al., 2013). Therefore, as described for other cellular models, stimulation of astrocytes with Thy-1/CD90 activates RhoA and Rac1 in a temporally inverse manner to modulate cytoskeleton rearrangements (Kong et al., 2013).

Because of its effects on integrins, Thy-1/CD90 is part of the integrin adhesome, as it regulates the integrin adhesion multimolecular complex that forms upon integrin activation. The adhesome is a complex formed by a large number of receptors and signaling molecules that govern the strength of adhesion and the dynamic turnover of Focal Adhesions (FAs) to

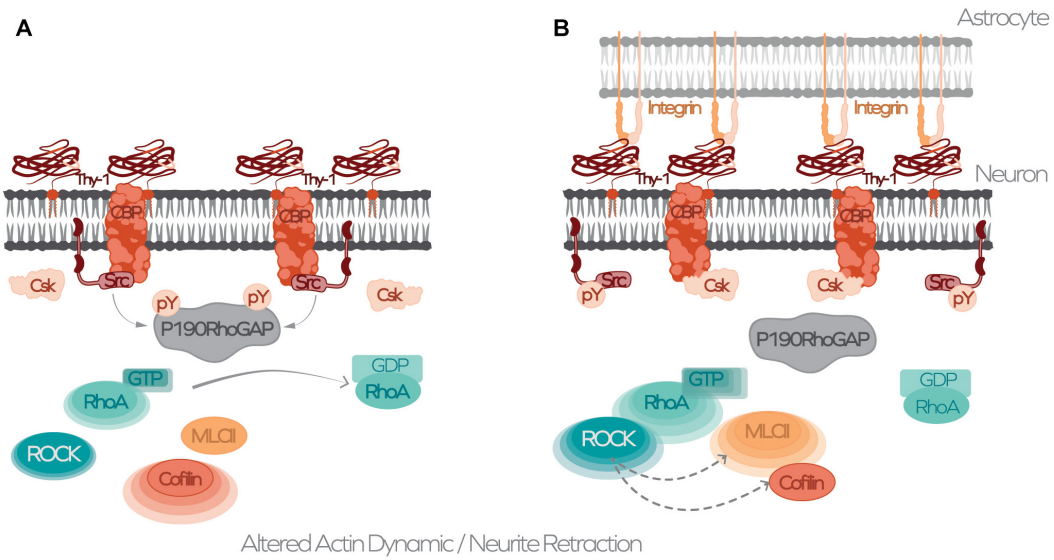

FIGURE 2 | Schematic model for Thy-1-dependent signaling and cytoskeleton regulation induced by $\alpha_v\beta_3$ integrin-binding. **(A)** Thy-1/CD90 nanoclusters at the neuronal plasma membrane dynamically associate or disassociate from CBP in lipid microdomains. Phosphorylated CBP serves as a docking site for active Src, which phosphorylates and activates p190RhoGAP that in turn activates the RhoA GTPase that hydrolyses GTP to GDP, inactivating RhoA. **(B)** Binding of $\alpha_v\beta_3$ integrin from astrocytes to Thy-1/CD90 induces clustering around CBP-Src-containing domains, recruiting Csk. Csk phosphorylates Src on Y527 and switches off Src activity. Inactive Src moves away from the Thy-1-CBP-Csk complex, inactivating p190RhoGAP. GAP inactivation increases RhoA activity, activating its effector ROCK, thereby leading to increased phosphorylation of cofilin and MLCII, and altering actin cytoskeleton dynamics.

regulate cell-ECM-attachment-detachment and movement. The structural core of the adhesome contains additional membrane proteins, including LRP1, SDC2, and SDC4 (Zaidel-Bar et al., 2007; Zaidel-Bar and Geiger, 2010). SDC4 associates with ECM proteins such as Fibronectin, which possesses three types of Fibronectin repeats containing an integrin-binding site (RGD tripeptide) and HepII (or Heparin-binding domain, HBD), that interact with the SDC4 heparan sulfate motifs. Through these integrin and SDC4 adhesion complexes, ECM proteins and their receptors control cyclic variations of these protein interactions, accounting for the dynamic changes of FAs that allow switching from strong cell adhesion to cell migration.

As mentioned above (**Figure 1**), Thy-1/CD90 has one binding site for $\alpha_v\beta_3$ integrin and another for SDC4. These binding motives are required for the Thy-1/CD90-stimulated formation of FA and stress fibers in astrocytes, which occurs within the first 20 min after stimulation (Avalos et al., 2009). Mutating Thy-1/CD90 in its HBD [Thy-1(AEAAA)] precludes Thy-1/CD90-induced activation of Rac1. In contrast, mutation of the Thy-1/CD90 RLD motif to RLE blocks Thy-1/CD90 binding to integrins, however, Thy-1/CD90 can still associate with SDC4 through its HBD and shows a tendency to increase Rac1 activity at 30 min of stimulation, whereas non-mutated Thy-1/CD90 inhibits Rac1 activity at this same time point [**Figure 3**, dashed purple lines; (Kong et al., 2013)]. These results support the idea that both integrin and SDC4 engagement by Thy-1/CD90 are required to induce Rac1 activation. They also support the existence of a trimolecular complex formed by the association of Thy-1/CD90 with $\alpha_v\beta_3$ integrin and SDC4, similar to the one described by Barker's group in melanoma cells, which controls

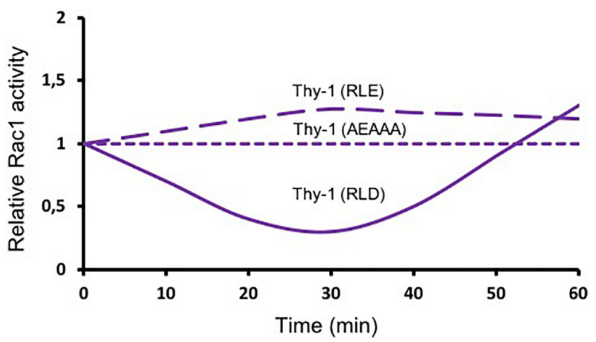

FIGURE 3 | Thy-1/CD90 binds to $\alpha_v\beta_3$ integrin and Syndecan-4 and activates Rac1. The astrocyte cell line DI TNC1 was incubated in serum-free medium and then stimulated with Thy-1-Fc-Protein A(RLD)-Sepharose beads (solid line), Thy-1 (RLE) or Thy-1 (AEAAA) (dashed lines) for different periods of time. A pull-down assay for active Rac was performed by affinity-precipitating the cell lysates. Total Rac1 from whole cell lysates and active Rac1 were visualized by immunoblotting with anti-Rac1 polyclonal antibody. Lines are a schematic representation of the results obtained in these experiments (Kong et al., 2013) indicating the fold-increase in Rac1 activity normalized to total protein present in the input lysate (Relative Rac1 activity).

the dynamic changes of FAs (Fiore et al., 2014). An interesting aspect of the Thy-1/CD90—integrin association is that, contrary to Fibronectin-integrin interaction -which occurs through a catch bond (force increase bond strength, slowing down dissociation)- Thy-1/CD90—integrin interaction occurs via a slip bond (force accelerates dissociation) for both $\alpha_v\beta_3$ (Burgos-Bravo et al., 2018) and $\alpha_5\beta_1$ integrins (Fiore et al., 2014). Here, the trimolecular

complex formed with SDC4 changes the slip bond of Thy-1/CD90—$\alpha_5\beta_1$ integrin to a catch bond. Whether the same is true for a potential Thy-1/CD90—$\alpha_V\beta_3$ integrin—SDC4 complex remains to be investigated. In any case, it is expected that an interplay between the downstream signaling pathways triggered downstream of $\alpha_v\beta_3$ integrin and SDC4 receptors occurs at least in mesenchymal type of cells (Morgan et al., 2007).

A remarkable feature of cell migration is the cyclic and dynamic turnover of points of adhesion, where after an initially induced strong adhesion, cells disassemble FAs, form cell protrusions and establish cell polarity, which increases the ability of cells to move. This dynamic process requires equally dynamic changes of the interactions taking place between Thy-1/CD90 and its binding partners. Thus, the lipid-protein composition of the microdomains where Thy-1/CD90 resides, and which are known to undergo constant, rapid, and dynamic organization [reviewed in Ilic et al. (2019)] are ideal platforms to allow the fluid and active arrangements of their components.

THY-1/CD90 IN CELL-TO-CELL INTERACTIONS

Recent reports have indicated that the interaction of Thy-1/CD90 with integrin mediates association of many different cell types [reviewed in Kong et al. (2013), Furlong et al. (2018), Hagood (2019), Hu and Barker (2019), Ilic et al. (2019), Morris (2018), and Picke et al. (2018b)]. For example, Thy-1/CD90 plays an important role as a β_3 integrin ligand in fibroblasts and cancer cells. In dermal fibroblasts, Thy-1/CD90 surface protein engages β_3 integrin *in trans* on adjacent cells, including fibroblasts or tumor cells. This interaction reportedly controls the balance between proliferation, apoptosis and differentiation, with a clear role in fibrosis, tissue repair and cancer progression (Schmidt et al., 2015). In hepatocarcinoma cells or cells from liver tumor tissue samples, expression of Thy-1/CD90 induces anchorage-independent growth and the expression of the stem cell marker, CD133. The effect of high levels of Thy-1/CD90 on CD133 expression has been described as dependent on the AMPK/mTOR signaling pathway and on the interaction of Thy-1/CD90 with β_3 integrin, since the effect of Thy-1/CD90 is abolished when the RLE mutant is expressed. Indeed, silencing of β_3 integrin *in vivo* abolishes tumor growth of CD90+ cells, and *in vitro*, CD133 is not expressed and the phosphorylation levels of mTOR and AMPK are not altered, suggesting a role for this interaction in hepatocarcinogenesis (Chen W. et al., 2015). Interestingly these authors have also indicated that Thy-1/CD90-β_3 integrin interaction inhibits ovarian cancer formation (Chen et al., 2016) and in this case, CD133 decreases its expression in cancer stem cells, while the phosphorylation of AMPK increases. In both articles, Thy-1/CD90 acts as a β_3 integrin ligand, however, in liver cancer cells, Thy-1/CD90 is presented as a carcinogenesis promoter, whereas in ovarian cancer, it is an inhibitor of cancer formation. Is there a third protein regulating the conformational state of Thy-1/CD90-integrin interaction to induce such different effects in cancer cells? How is Thy-1/CD90 able to perform "hero and villain" functions and act as tumor suppressor or tumor promoter, respectively, using the same molecular signaling pathways? Is it possible that *cis* versus *trans* interactions account for these two distinct roles of Thy-1/CD90-integrin binding?

Additionally, Thy-1/CD90 expressed in activated endothelial cells at sites of inflammation binds to monocytes, leukocytes, and melanoma cells through various integrins, such as $\alpha_V\beta_3$, $\alpha_X\beta_2$, or $\alpha_M\beta_2$ integrins (Wen et al., 2013) and also via the seven-transmembrane protein CD97, possibly to allow *trans*-endothelial migration of these cells (Ward et al., 2018). The interaction of Thy-1/CD90 with CD97 was reported in 2012 (Wandel et al., 2012), when it was known that CD97 could also interact with $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Wang et al., 2005), CD55 (Hamann, 2004), and chondroitin sulfate glycosaminoglycans (Stacey et al., 2003). The association of CD97 with the integrins is intriguing, particularly because direct binding of Thy-1/CD90 with CD97 was demonstrated in assays where either pure soluble proteins or a pure protein-to-cell binding were used; in all cases the interactions were only partially inhibited by antibodies directed to any of these two molecules (Wandel et al., 2012). The question that arises here is whether the effect observed in cell-to-cell binding experiments is actually mediated by a *trans* association of Thy-1/CD90 with CD97, or of CD97 with $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins; the latter regulated by a *cis* Thy-1/CD90 association with inactive integrins. Importantly, both cells used in Wandel's study, activated endothelial and CHO cells, express integrins (Kim et al., 2002; Ward et al., 2018). Nowadays, many studies keep reporting CD97/integrin interaction (Ward et al., 2018; Tjong and Lin, 2019), whereas no recent evidence about binding of CD97 to Thy-1/CD90 has been reported after Wandel's description in 2012, supporting the hypothesis that Thy-1/CD90 might be a regulatory element of the CD97/integrin interaction through the regulation of integrin activity. Such mode of regulation was recently reported by Fiore et al. (2015) in a different cellular model. Using fibroblasts, Barker's group described that conformational coupling between integrins and Thy-1/CD90 in a *cis* interaction, changes the avidity of integrins for ECM proteins, thus regulating integrin-ECM interactions and fibroblast response to ECM proteins (Fiore et al., 2015; Hagood, 2019; Hu and Barker, 2019). Therefore, Thy-1/CD90—$\alpha_v\beta_3$ integrin *in cis* could regulate integrin interaction with CD97 *in trans*, in a similar manner.

THY-1/CD90-INTEGRIN ASSOCIATION AND SIGNALING UNDER INFLAMMATORY SIGNALS

In chronic inflammatory diseases, such as psoriasis, Thy-1/CD90 is highly expressed in skin lesions, and experiments performed with polymorphonuclear (PMN) cells from psoriatic patients have shown that Thy-1/CD90 is involved in the process of adhesion of these cells to activated endothelial cells in an $\alpha_M\beta_2$ integrin (CD11b/CD18; Mac-1)-dependent manner (Wetzel et al., 2006). Reports also indicate that the Thy-1/CD90-$\alpha_M\beta_2$ integrin interaction is an important mediator of transendothelial migration through cytokine-activated endothelium and therefore, it would also play an

important role in leukocyte invasion into inflamed tissues (Haustein et al., 2014). Thy-1/CD90 expression is elevated in endothelial cells exposed to pro-inflammatory cytokines, such as IL-1β and TNFα (Haustein et al., 2014). Interestingly, these two cytokines are up regulated in psoriasis (Arican et al., 2005) and neutrophils from these patients adhere more to endothelial cells than those from healthy donors (Wetzel et al., 2006). In addition, Thy-1/CD90 binding to neutrophils triggers the secretion of MMP-9 and CXCL8, facilitating the transport of these cells to the lesion (Saalbach et al., 2008). Thus, under inflammatory conditions, Thy-1/CD90 enhances its expression levels in endothelial cells and mediates adhesion and migration of PMN cells in an integrin-dependent manner. Additionally, the Thy-1/CD90-$\alpha_M\beta_2$ integrin interaction also regulates neutrophil function, allowing them to not only recognize the affected tissue, but also to quickly arrive to the site of inflammation.

In other inflammatory diseases such as rheumatoid arthritis, fibroblasts also show overexpressed Thy-1/CD90 levels in cells located at the inflamed synovium. These fibroblasts are proliferative, invasive and produce pro-inflammatory cytokines and expand three times more than those fibroblasts found in osteoarthritis patients (Mizoguchi et al., 2018). Likewise, in systemic sclerosis, another inflammatory disease of the skin, altered fibroblasts present high Thy-1/CD90 levels (Nazari et al., 2016). Additionally, in cancer associated fibroblasts, Thy-1/CD90 induces inflammation and increases tumor progression by promoting IL-6 secretion (Shiga et al., 2015; Huynh et al., 2016). However, in lung cystic fibrosis, Thy-1/CD90 negative fibroblasts are more migratory and contribute to the formation of the fibrotic tissue (Hagood et al., 2005). Thus, in the case of fibroblasts, Thy-1/CD90 expression under inflammatory conditions seems to be related to bad prognosis in only some specific settings.

On the other hand, Thy-1/CD90 is considered a mesenchymal stem cell (MSC) marker, and MSCs with high levels of Thy-1/CD90 are thought to regulate the immune response since MSCs with decreased Thy-1/CD90 levels have been associated with loss of their immunosuppressor activity (Campioni et al., 2009). Thy-1/CD90 is increased in inflammatory diseases such as periodontitis and in this case, MSCs with elevated levels of Thy-1/CD90 contribute to the immunosuppressive environment that controls the inflammation (Estrela et al., 2017). In the brain, there is a mesenchymal cell population that overexpresses Thy-1/CD90 near the microvasculature associated to the blood-brain barrier, which reduces the inflammatory response compared with a lower Thy-1/CD90-expressing population (Park et al., 2016). In a model of rat intracerebral hemorrhage, intravenous transplantation of MSCs reduces the disruption of the blood-brain barrier by decreasing migration of microglia and PMN cells, increasing the levels of anti-inflammatory cytokines and thereby leading to an attenuated inflammatory response (Chen M. et al., 2015). Similar results have been obtained in a rat traumatic brain injury model, where MSCs were transplanted 2 h after the injury (Zhang et al., 2013). Of note, the MSCs used for transplantation are characterized by their surface marker expression, which includes high levels of Thy-1/CD90. Moreover, MSCs from adipose tissue and bone marrow of canine origin have been compared, and although cells from adipose tissue show higher DNA methylation and proliferative rate than those from bone marrow, the immunosuppressive properties of both MSC types are similar. Importantly, both types of MSCs showed increased levels of Thy-1/CD90 (Russell et al., 2016). Thus, MSCs have a recognized protective effect in various disorders by modulating the inflammatory response; this feature seems to be associated with elevated expression of Thy-1/CD90. However, further studies are needed to confirm this correlation.

Interestingly, in the neuron-astrocyte model, Thy-1/CD90 needs to be in an inflammatory environment to be functional, since its receptors are only expressed in sufficient amounts and conformation in astrocytes that exhibit a reactive phenotype (Lagos-Cabré et al., 2017). Rat astrocytes derived from neonate animals only respond to Thy-1/CD90 when reactivated by pro-inflammatory cytokines such as TNF (Lagos-Cabré et al., 2017). Reactive astrocytes obtained from transgenic neonatal mice that carry the human superoxide dismutase mutated in glycine 93 ($hSOD^{G93A}$) also respond to Thy-1/CD90. These mice develop amyotrophic lateral sclerosis (ALS) at 3 months of age; thus, they constitute a mouse model for this neurodegenerative disease (Van Zundert et al., 2012). Interestingly, astrocytes derived from $hSOD^{G93A}$ mouse brains at postnatal days 1–2, and cultured *in vitro* for 3–4 weeks behave as reactive astrocytes (contrary to the transgenic wild type hSOD mouse-derived astrocytes), confirming that these cells are reactive before the onset of the disease symptoms. Considering that astrocytes treated with pro-inflammatory cytokines or derived from the transgenic $hSOD^{G93A}$ mice both show a reactive phenotype, and increased expression of integrin and SDC4 protein (Lagos-Cabré et al., 2017), it is possible that Thy-1/CD90 requires the expression of these receptors at levels where effective interactions might take place in order to trigger downstream signaling pathways. We speculate then that Thy-1/CD90 is incapable of activating $\alpha_v\beta_3$ integrin and SDC4 within intact and healthy tissue because the expression levels of these receptors are too low, but rise locally in damaged areas or where pro-inflammatory conditions are present.

TNF treatment of astrocytes, apart from inducing elevation of cell surface proteins such as $\alpha_v\beta_3$ integrin and SDC4, additionally elevates Connexin 43, Pannexin 1, and the purinergic receptor P2X7R levels; despite these changes, TNF does not stimulate cell migration unless the receptors are engaged by Thy-1/CD90 (Lagos-Cabré et al., 2017, 2018). However, by increasing the amount of these proteins at the plasma membrane, TNF prepares the cells to respond to Thy-1/CD90 by allowing the formation of integrin microclusters, and possibly of SDC4 (Lagos-Cabré et al., 2018). A similar effect is achieved by overexpressing β_3 integrin in the absence of TNF; in this case, β_3 integrin-expressing cells are also primed to respond to Thy-1/CD90 by generating receptor microclusters in the membrane, which become more prominent and effective upon Thy-1/CD90 association with its receptors (Lagos-Cabré et al., 2017). Therefore, it appears that the receptors need to reach a certain level of expression/aggregation to prepare the cell to respond to the neuronal ligand Thy-1/CD90.

Results reported in rat brain-derived astrocytes show that ATP release and Ca^{2+} uptake via the P2X7R are key steps in astrocyte migration induced by Thy-1/CD90, which coincides with the

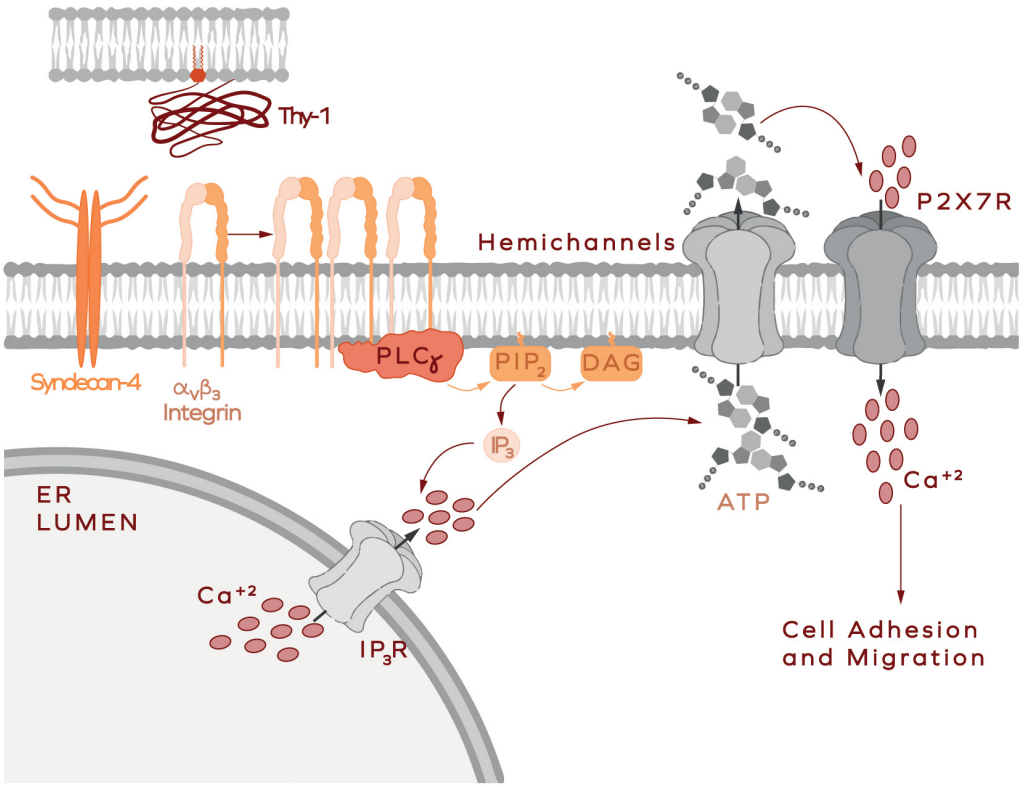

FIGURE 4 | Thy-1-induced astrocyte adhesion and migration. In astrocytes, Thy-1/CD90 binding to integrin microclusters induces receptor oligomerization and leads to the formation of bigger clusters and integrin activation. Integrin then recruits signaling molecules, such as PLCγ. Active PLCγ hydrolyzes PIP_2, generating diacylglycerol (DAG) and IP_3. The latter activates the inositol 1,4,5-trisphosphate receptor (IP_3R) to allow the release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum (ER lumen is shown). Increased intracellular Ca^{2+} concentration opens the hemichannels (Connexin 43 and Pannexin 1), leading to the consequent release of ATP to the extracellular medium. Locally increased ATP concentration activates P2X7 receptors, which allow Ca^{2+} entry into the cell. This signaling pathway is part of a more complex cascade of reactions leading to cell polarization, adhesion and migration [more details in Alvarez et al. (2016) and Lagos-Cabré et al. (2018)].

signaling mechanisms involved in cell adhesion and migration of DITNC1 astrocytes (Henriquez et al., 2011; Alvarez et al., 2016; Lagos-Cabré et al., 2017, 2018). The signaling pathways include, the activation of FAK/Src/PI3K and PLCγ, the generation of the second messengers DAG and IP_3, the activation of the IP_3R and the release of Ca^{2+} from intracellular stores, consequently increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Avalos et al., 2009; Kong et al., 2013; Alvarez et al., 2016). The elevated Ca^{2+} levels lead to the opening of the Connexin 43 and Pannexin 1 hemichannels, and the release of ATP to the extracellular space. Increased ATP levels activate the P2X7R, allowing the entrance of extracellular Ca^{2+}, further rising ($[Ca^{2+}]_i$) (**Figure 4**; Alvarez et al., 2016). Thy-1/CD90-stimulated astrocyte adhesion and migration could be precluded by hydrolyzing ATP with Apyrase treatment, chelating extracellular Ca^{2+} with EGTA, or by silencing/inhibiting P2X7R pharmacologically. This complex signaling cascade required for cells to undergo migration, relies heavily on the increase of ($[Ca^{2+}]_i$) (Alvarez et al., 2016; Lagos-Cabré et al., 2017, 2018). However, contrary to intuition, and although an ionophore stimulates cells to open hemichannels and release ATP, the treatment of primary cells with a Ca^{2+} ionophore to bypass the membrane receptors' downstream signaling does not induce astrocyte migration. Instead, cell motility requires that pro-inflammatory stimuli are added (e.g., TNF) (Lagos-Cabré et al., 2018). These results imply that parallel signaling pathways activated by Thy-1/CD90 binding to its receptors, and the fine-tune regulation of the levels of these receptors by a pro-inflammatory environment, are important factors of the signal transduction pathways that these cells utilize to move.

Therefore, Thy-1/CD90 cell surface glycoprotein possesses various functions that depend on the context of specific physiological or pathological conditions. Such conditions are determined by inflammatory processes, expression levels of Thy-1/CD90, as well as the expression levels of its binding counterparts.

CELLULAR RESPONSES REGULATED BY THY-1/CD90 *IN CIS*

Theoretical and experimental evidence indicate that Thy-1/CD90 interacts with several proteins. Interestingly, the interactions occur with intracellular or extracellular proteins, with molecules located at the cell surface of the same cells, and with proteins

present in other cells. Despite being a GPI-anchored protein that resides on the outer leaflet of the plasma membrane, this protein can modulate signal transduction pathways through the lipid bilayer to the interior of the cell, and regulate processes like apoptosis, differentiation, proliferation, and tumor suppression (Rege and Hagood, 2006). This highlights the role of Thy-1/CD90 in the same cell that expresses this protein; i.e., "*cis signaling*," in addition to the effect that this ligand exerts in other cells ("*trans signaling*").

The mechanism by which Thy-1/CD90 can transduce signals to the interior of a cell has been elucidated in fibroblasts and neurons, and involves the participation of the CBP scaffold protein as a transducer (Chen et al., 2009; Maldonado et al., 2017). In neurons, integrin stimulation promotes Thy-1/CD90 clustering and the formation of a complex with CBP, Csk, and Src. Csk phosphorylates Src in a C-terminal inactivating tyrosine (Y527), thereby leading to the separation of Src from the complex (**Figure 2**). The inactivation of Src inhibits the RhoGAP activity of p190RhoGAP, which is one of the main substrates of Src in neurons (Brouns et al., 2001). As a consequence, GTP-coupled RhoA cannot be hydrolysed and its activity increases, leading to contraction of the actin cytoskeleton and the resultant retraction of neuronal processes (Maldonado et al., 2017). Evidence has indicated that in fibroblasts, CBP also plays a key role in the transient confinement of Thy-1/CD90 clusters in lipid rafts, however, this study focused on deciphering how a GPI-anchored protein could undergo patching and capping if it cannot interact directly with the cytoskeleton. Here, Jacobson's group described that by binding to the adaptor protein EBP50, CBP links the Thy-1/CD90/CBP complex to the actin cytoskeleton through an ezrin-radixin-moesin protein complex (Chen et al., 2009). Therefore, transient anchorage of Thy-1/CD90 in lipid rafts induces signaling amongst proteins that do not span the whole lipid bilayer, confining in one place receptors, scaffolds, adaptors and signaling molecules to promote cell signaling.

The *cis* binding of Thy-1/CD90 has not only been reported for Thy1-Thy1 interaction in cluster formation, but is now well documented for integrins, which are its classical counter receptors. The binding between Thy-1/CD90 and integrins has been widely documented in *trans signaling* (Barker and Hagood, 2010), but more recent evidence has additionally incorporated the concept of Thy-1/CD90 *cis signaling*. In lung fibrosis, Thy-1/CD90 associates with $\alpha_v\beta_5$ integrin in *cis*, promoting the inhibition of latent TGF-β1 activation induced by myofibroblast contraction, likely by competing with the RGD motifs on the N-terminal latency-associated peptide (LAP) and those of ECM proteins (Zhou et al., 2010). Another example is that of the Thy-1/CD90-$\alpha_v\beta_3$ integrin duo in fibroblasts, which plays a crucial role in FA formation. In this context, Thy-1/CD90 *cis* binding to inactive $\alpha_v\beta_3$ integrin acts as a sensor of matrix rigidity and controls the association of this integrin with ECM proteins, as well as with critical lipid raft components such as Fyn and CBP. This interaction controls c-Src activity and thus modulates FA formation dynamics (Fiore et al., 2015). The regulatory role of Thy-1/CD90 on $\alpha_v\beta_3$ integrin avidity for the ECM has been recently corroborated in an *in vivo* model of fibrotic lung injury (Fiore et al., 2018). This *cis* interaction has been particularly analyzed for $\alpha_v\beta_3$ integrin in ovary and liver cancer cells, where in both cases *cis* Thy-1/CD90-$\alpha_v\beta_3$ integrin interaction modulates cancer progression. Intriguingly, the effect of Thy-1/CD90 presence is opposite in these cancer types; behaving as a tumor promoter in liver, but as a tumor suppressor in ovarian cancer. Even more surprising is the fact that the AMPK/mTOR/CD133 signaling axis has been implicated in both cases (Chen W. et al., 2015; Chen et al., 2016). Here, *cis* (regulating) versus *trans* (activating) interactions could be mediating the opposite cellular responses. Therefore, *cis* interaction of Thy-1/CD90 with integrins seems to play a regulatory role, in which the outcome is cell context-dependent.

Beyond the interaction of Thy-1/CD90 with the classical counterpart proteins, reports indicate that it also associates in *cis* with other membrane components, such as ion channels and transmembrane receptors. For example, in the adult rat retina, Thy-1/CD90 colocalizes with the ion channel subunit 4 of the hyperpolarization-activated, cyclic nucleotide-gated ("HCN") protein (Partida et al., 2012). The authors suggest a new possible electrophysiological property for Thy-1/CD90, which would reveal the versatile nature of this protein in other settings as well. On the other hand, Thy-1/CD90 association with the FasR has also been documented in lung myofibroblasts as a key requisite for cell apoptosis. The absence of Thy-1/CD90 in these cells decreases apoptosis and thus, tissue regeneration upon lung injury cannot be completed, thereby leading to a progressive fibrotic disorder (Liu et al., 2017). Experiments performed in these studies to demonstrate the association of Thy1/CD90 with these proteins include immunoprecipitation and colocalization by confocal microscopy, however, given the known limitations of these techniques, the direct interaction of Thy-1/CD90 with either HCN4 or FasR has not been confirmed yet. In any case, either directly or indirectly, the HCN4 subunit and FasR are Thy-1/CD90 partners that further increase the plethora of molecules that might associate with Thy-1/CD90.

EXTRACELLULAR THY-1/CD90-LINKED PLASMA MEMBRANE-ASSOCIATED PLATFORM

As a concept, a scaffold protein is defined as a multivalent molecule that integrates a diverse set of other components in a spatial and temporal manner. These components are part of a distinct signaling pathway, but their proximity might activate other pathways, increasing the biological activities or processes carried out by the cell. Moreover, the crowding property gives these scaffold proteins a central role in the physical assembly of different molecular components to enhance the specificity and the efficiency of various signal transduction pathways. Initially, it was thought that a scaffold is limited to regulating the proximity of certain enzymes, but today its function expands to a much more complex scenario of regulation, with structural and functional plasticity (Good et al., 2011; Pan et al., 2012).

Within this scaffold concept, and considering Thy-1/CD90 physicochemical properties, binding capabilities, and signal transducing functions, we can place Thy-1/CD90 as an

integrating and organizer molecule at the extracellular level. Thy-1/CD90 binds molecules that act as transmembrane transducers, which are collectively named transmembrane adaptor proteins (TRAPs), such as LAT and CBP, both of which transduce the signaling triggered by Thy-1/CD90 binding to the interior of the cell (Leyton et al., 1999; Maldonado et al., 2017). These TRAPs have two palmitoylation sites and by virtue of these lipid modifications, are located in rafts with little mobility (Zhang et al., 1998). Thus, by binding to these palmitoylated transmembrane proteins, Thy-1/CD90 becomes confined and less mobile within the microdomains.

Thy-1/CD90 additionally associates with various membrane receptors. It binds to various integrins, SDC4, FasR, HCN4, and CD97, controlling in each case, different cellular components and events. Thus, the variety of binding partners that Thy-1/CD90 displays and its ability to regulate the function of other proteins, makes it a surface scaffold candidate, much like the GPI-anchored protein PrPC has been postulated as an extracellular scaffold (Linden, 2017). However, the scene is more complex, because the Thy-1/CD90 scaffold function occurs both *in cis* and *in trans*. For example, Thy-1/CD90 binds to integrins in the plane of the membrane to maintain it in an inactive state. However, when Thy-1/CD90-integrin interaction occurs between opposing cells, integrins behave as signaling scaffolds themselves and engage components of the cytoskeleton and the ECM to regulate various cellular processes (LaFlamme et al., 2018). The mechanism is even more complex, because Thy-1/CD90 is located in lipid rafts, and lipid composition determines different types of rafts. Thy-1/CD90 locates in rafts that associate with actin and possess saturated lipids, whereas PrPC is in microdomains with more unsaturated and longer chain lipids (Brügger et al., 2004), which do not contact the actin cytoskeleton (Chen et al., 2008;

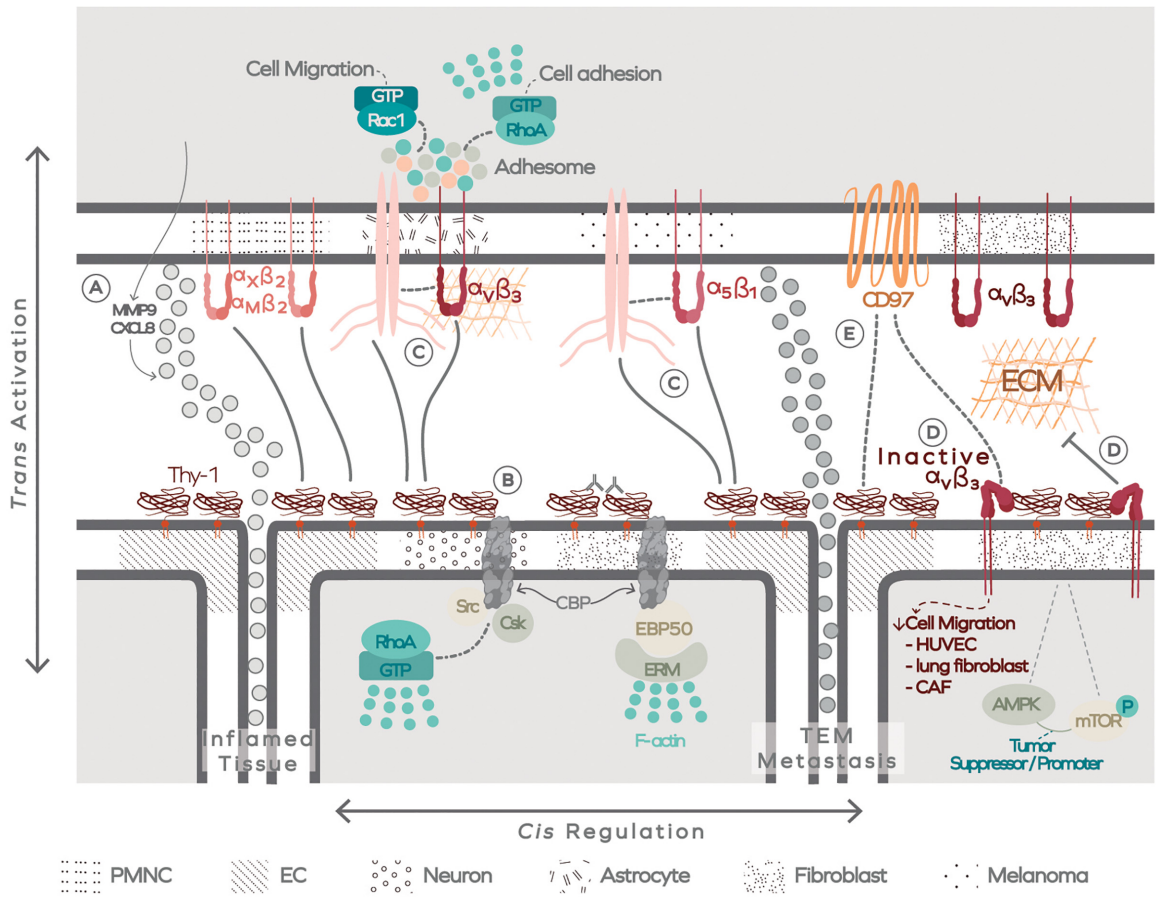

FIGURE 5 | The extracellular Thy-1-linked membrane-associated platform (ThyMAP). Data has been obtained in different cells, as indicated by different textures in the plasma membrane. Thy-1/CD90 binding to integrin occurs *in trans* (activating) and *in cis* (regulating). **(A)** Thy-1/CD90 binding to PMN cells through $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins promotes transendothelial migration of these cells, facilitating their arrival to the inflamed tissue. The interaction also induces production of MMP9 and CXCL8. **(B)** In addition, Thy-1/CD90 crosslinking with antibodies or by binding to $\alpha_v\beta_3$ integrin *in trans* forms Thy-1/CD90 nanoclusters and signals to the interior of the cell through the transmembrane adaptor CBP, which transiently confines Thy-1/CD90 by engaging the actin cytoskeleton. **(C)** Thy-1/CD90 also forms a ternary complex with $\alpha_5\beta_1$ integrin, and possibly with $\alpha_v\beta_3$, to facilitate melanoma or astrocyte migration, respectively. **(D)** The regulatory role of Thy-1/CD90 appears to occur in a *cis* interaction with $\alpha_v\beta_3$ integrin. This *cis* interaction decreases integrin avidity for ECM proteins and could regulate Thy-1/CD90 tumor suppressor or tumor promoter activity and, additionally, control the interaction of integrins with CD97. **(E)** The latter could also interact directly with Thy-1/CD90. Polymorphonuclear cells (PMNC); Endothelial cells (EC); Transendothelial migration (TEM).

Morris et al., 2011). Additionally, lipid and protein composition of these raft structures is dynamic and arranged in nanodomains that rapidly reorganize and change their components to control signal transduction events [reviewed in Ilic et al. (2019)].

Astro and De Curtis (2015) have coined a new concept for these "dynamic scaffolds that organize membrane-associated events" that is Plasma membrane-associated platforms or PMAPs. However, these platforms are a combination of scaffold proteins that associate with membrane receptors within the cell and contain a core complex (formed by Liprin, ELK, CLASP, LL5). Although this core complex participates in processes like cell adhesion, migration, and leading edge protrusive activity, they have not been involved -up to now- in Thy-1/CD90 signaling. Importantly, integrins are active participants of PMAPs (LaFlamme et al., 2018). Thus, much still needs to be learnt about Thy-1/CD90 interactions and signaling mechanisms regulated by these associations, and it is possible that an extracellular Thy-1-linked membrane-associated platform (ThyMAP) represents a different supramolecular assembly where Thy-1/CD90, centered at its core, organizes various signal transduction pathways from the extracellular molecular platform (**Figure 5**).

In summary, Thy-1/CD90 can interact with itself forming clusters that bind to different transmembrane proteins. These proteins connect with the ECM, intracellular molecules, the cytoskeleton, and other membrane receptors present in the same or neighboring cells. Therefore, Thy-1/CD90 forms clusters, supramolecular complexes, and participates in the activation (*in trans*) and regulation (*in cis*) of different signal transduction pathways. Such signaling reactions are restricted by the surface proteins expressed in the cells and their surrounding microenvironment. Therefore, the multimeric complexes formed with Thy-1/CD90 as a key organizer of intra and extracellular scaffolds, spatiotemporally control specificity and efficiency of the cellular responses.

AUTHOR CONTRIBUTIONS

JD, SM, EP, LAP, and RDP wrote the different sections of the manuscript. JD organized the citations and performed the graph scheme. LL performed the conception, design, and wrote the manuscript. All authors contributed to manuscript revision and approved the submitted version of the manuscript.

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REFERENCES

Alvarez, A., Lagos-Cabré, R., Kong, M., Cárdenas, A., Burgos-Bravo, F., Schneider, P., et al. (2016). Integrin-mediated transactivation of P2X7R via hemichannel-dependent ATP release stimulates astrocyte migration. *Biochim. Biophys. Acta* 1863, 2175–2188. doi: 10.1016/j.bbamcr.2016.05.018

Arican, O., Aral, M., Sasmaz, S., and Ciragil, P. (2005). Serum levels of TNF-α, IFN-γ, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. *Mediators Inflamm.* 2005, 273–279. doi: 10.1155/mi.2005.273

Astro, V., and De Curtis, I. (2015). Plasma membrane-associated platforms: dynamic scaffolds that organize membrane-associated events. *Sci. Signal.* 8:re1. doi: 10.1126/scisignal.aaa3312

Avalos, A. M., Arthur, W. T., Schneider, P., Quest, A. F. G., Burridge, K., and Leyton, L. (2004). Aggregation of integrins and RhoA activation are required for Thy-1-induced morphological changes in astrocytes. *J. Biol. Chem.* 279, 39139–39145. doi: 10.1074/jbc.M403439200

Avalos, A. M., Valdivia, A. D., Munoz, N., Herrera-Molina, R., Tapia, J. C., Lavandero, S., et al. (2009). Neuronal Thy-1 induces astrocyte adhesion by engaging syndecan-4 in a cooperative interaction with αvβ3 integrin that activates PKCα and RhoA. *J. Cell Sci.* 122, 3462–3471. doi: 10.1242/jcs.034827

Barker, T. H., and Hagood, J. S. (2010). Getting a Grip on Thy-1 Signaling. *Biochim. Biophys. Acta* 1793, 921–923. doi: 10.1016/j.bbamcr.2008.10.004s

Boulter, E., Garcia-Mata, R., Guilluy, C., Dubash, A., Rossi, G., Brennwald, P. J., et al. (2010). Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. *Nat. Cell Biol.* 12, 477–483. doi: 10.1038/ncb2049

Brouns, M. R., Matheson, S. F., and Settleman, J. (2001). P190 RhoGAP is the principal Src substrate in brain and regulates axon outgrowth, guidance and fasciculation. *Nat. Cell Biol.* 3, 361–367. doi: 10.1038/35070042

Brügger, B., Graham, C., Leibrecht, I., Mombelli, E., Jen, A., Wieland, F., et al. (2004). The membrane domains occupied by glycosylphosphatidylinositol-anchored prion protein and thy-1 differ in lipid composition. *J. Biol. Chem.* 279, 7530–7536. doi: 10.1074/jbc.M310207200

Burgos-Bravo, F., Figueroa, N. L., Casanova-Morales, N., Quest, A. F. G., Wilson, C. A. M., and Leyton, L. (2018). Single-molecule measurements of the effect of force on Thy-1/αvβ3-integrin interaction using nonpurified proteins. *Mol. Biol. Cell* 29, 326–338. doi: 10.1091/mbc.e17-03-0133

Burridge, K., and Wennerberg, K. (2004). Rho and Rac Take Center Stage. *Cell* 116, 167–179. doi: 10.1016/s0092-8674(04)00003-0

Campioni, D., Rizzo, R., Stignani, M., Melchiorri, L., Ferrari, L., Moretti, S., et al. (2009). A decreased positivity for CD90 on human mesenchymal stromal cells (MSCs) is associated with a loss of immunosuppressive activity by MSCs. *Cytom. B Clin. Cytom.* 76, 225–230. doi: 10.1002/cyto.b.20461

Chen, M., Li, X., Zhang, X., He, X., Lai, L., Liu, Y., et al. (2015). The inhibitory effect of mesenchymal stem cell on blood-brain barrier disruption following intracerebral hemorrhage in rats: Contribution of TSG-6. *J. Neuroinflammation* 12:61. doi: 10.1186/s12974-015-0284-x

Chen, W., Chang, Y., Hsu, H., Yen, M., Cho, C., Wang, C., et al. (2015). Therapeutics targeting CD90-integrin-AMPK-CD133 signal axis in liver cancer. *Oncotarget* 6, 42923–42937. doi: 10.18632/oncotarget.5976

Chen, W., Hsu, H., Li, C., Yang, Y., Hung, Y., Cho, C., et al. (2016). Cancer stem cell marker CD90 inhibits ovarian cancer formation via β3 integrin. *Int. J. Oncol.* 49, 1881–1889. doi: 10.3892/ijo.2016.3691

Chen, X., Jen, A., Warley, A., Lawrence, M. J., Quinn, P. J., and Morris, R. J. (2008). Isolation at physiological temperature of detergent-resistant membranes with properties expected of lipid rafts: the influence of buffer composition. *Biochem. J.* 417, 525–533. doi: 10.1042/bj20081385

Chen, Y., Veracini, L., Benistant, C., and Jacobson, K. (2009). The transmembrane protein CBP plays a role in transiently anchoring small clusters of Thy-1, a GPI-anchored protein, to the cytoskeleton. *J. Cell Sci.* 122, 3966–3972. doi: 10.1242/jcs.049346

Estrela, C., Freitas Silva, B. S., Silva, J. A., Yamamoto-Silva, F. P., Pinto-Júnior, D. D., and Gomez, R. S. (2017). stem cell marker expression in persistent apical periodontitis. *J. Endod.* 43, 63–68. doi: 10.1016/j.joen.2016.09.002

Fiore, V. F., Ju, L., Chen, Y., Zhu, C., and Barker, T. H. (2014). Dynamic catch of a Thy-1-α5β1 +syndecan-4 trimolecular complex. *Nat. Commun.* 5:4886. doi: 10.1038/ncomms5886

Fiore, V. F., Strane, P. W., Bryksin, A. V., White, E. S., Hagood, J. S., and Barker, T. H. (2015). Conformational coupling of integrin and Thy-1 regulates Fyn priming and fibroblast mechanotransduction. *J. Cell Biol.* 211, 173–190. doi: 10.1083/jcb.201505007

Fiore, V. F., Wong, S. S., Tran, C., Tan, C., Xu, W., Sulchek, T., et al. (2018). αvβ3 Integrin drives fibroblast contraction and strain stiffening of soft provisional matrix during progressive fibrosis. *JCI Insight* 3:e97597. doi: 10.1172/jci.insight.97597

Furlong, S., Coombs, M. R. P., Ghassemi-Rad, J., and Hoskin, D. W. (2018). Thy-1 (CD90) signaling preferentially promotes RORγt expression and a Th17 response. *Front. Cell Dev. Biol.* 6:158. doi: 10.3389/fcell.2018.00158

Good, M. C., Zalatan, J. G., and Lim, W. A. (2011). Scaffold proteins: Hubs for controlling the flow of cellular information. *Science* 332, 680–686. doi: 10.1126/science.1198701

Hagood, J. S. (2019). Thy-1 as an integrator of diverse extracellular signals. *Front. Cell Dev. Biol.* 7:26. doi: 10.3389/fcell.2019.00026

Hagood, J. S., Prabhakaran, P., Kumbla, P., Salazar, L., MacEwen, M. W., Barker, T. H., et al. (2005). Loss of fibroblast Thy-1 expression correlates with lung fibrogenesis. *Am. J. Pathol.* 167, 365–379. doi: 10.1016/s0002-9440(10)62982-3

Hamann, J. (2004). The seven-span transmembrane receptor CD97 has a cellular ligand (CD55. DAF). *J. Exp. Med.* 184, 1185–1189. doi: 10.1084/jem.184.3.1185

Haustein, U.-F., Anderegg, U., Sticherling, M., Preissner, K. T., Saalbach, A., Chavakis, T., et al. (2014). Human Thy-1 (CD90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J. Immunol.* 172, 3850–3859. doi: 10.4049/jimmunol.172.6.3850

Henriquez, M., Herrera-Molina, R., Valdivia, A., Alvarez, A., Kong, M., Munoz, N., et al. (2011). ATP release due to Thy-1-integrin binding induces P2X7-mediated calcium entry required for focal adhesion formation. *J. Cell Sci.* 124, 1581–1588. doi: 10.1242/jcs.073171

Hermosilla, T., Muñoz, D., Herrera-Molina, R., Valdivia, A., Muñoz, N., Nham, S. U., et al. (2008). Direct Thy-1/αVβ3 integrin interaction mediates neuron to astrocyte communication. *Biochim. Biophys. Acta* 1783, 1111–1120. doi: 10.1016/j.bbamcr.2008.01.034

Herrera-Molina, R., Frischknecht, R., Maldonado, H., Seidenbecher, C. I., Gundelfinger, E. D., Hetz, C., et al. (2012). Astrocytic αVβ3 integrin inhibits neurite outgrowth and promotes retraction of neuronal processes by clustering thy-1. *PLoS One* 7:e34295. doi: 10.1371/journal.pone.0034295

Herrera-Molina, R., Valdivia, A., Kong, M., Alvarez, A., Cárdenas, A., Quest, A. F. G., et al. (2013). Thy-1-interacting molecules and cellular signaling in Cis and Trans. *Int. Rev. Cell Mol. Biol.* 305, 163–216. doi: 10.1016/B978-0-12-407695-2.00004-4

Hu, P., and Barker, T. H. (2019). Thy-1 in integrin mediated mechanotransduction. *Front. Cell Dev. Biol.* 7:22. doi: 10.3389/fcell.2019.00022

Huynh, P. T., Beswick, E. J., Coronado, Y. A., Johnson, P., O'Connell, M. R., Watts, T., et al. (2016). CD90+ stromal cells are the major source of IL-6, which supports cancer stem-like cells and inflammation in colorectal cancer. *Int. J. Cancer* 138, 1971–1981. doi: 10.1002/ijc.29939

Ilic, K., Auer, B., Mlinac-Jerkovic, K., and Herrera-Molina, R. (2019). Neuronal signaling by Thy-1 in nanodomains with specific ganglioside composition: shall we open the door to a new complexity? *Front. Cell Dev. Biol.* 7:27. doi: 10.3389/fcell.2019.00027

Kim, S., Bakre, M., Yin, H., and Varner, J. A. (2002). Inhibition of endothelial cell survival and angiogenesis by protein kinase A. *J. Clin. Invest.* 110, 933–941. doi: 10.1172/JCI0214268

Kong, M., Muñoz, N., Valdivia, A., Alvarez, A., Herrera-Molina, R., Cárdenas, A., et al. (2013). Thy-1-mediated cell-cell contact induces astrocyte migration through the engagement of αVβ3 integrin and syndecan-4. *Biochim. Biophys. Acta* 1833, 1409–1420. doi: 10.1016/j.bbamcr.2013.02.013

LaFlamme, S. E., Mathew-Steiner, S., Singh, N., Colello-Borges, D., and Nieves, B. (2018). Integrin and microtubule crosstalk in the regulation of cellular processes. *Cell. Mol. Life Sci.* 75, 4177–4185. doi: 10.1007/s00018-018-2913-x

Lagos-Cabré, R., Alvarez, A., Kong, M., Burgos-Bravo, F., Cárdenas, A., Rojas-Mancilla, E., et al. (2017). αVβ3 Integrin regulates astrocyte reactivity. *J. Neuroinflammation* 14:194. doi: 10.1186/s12974-017-0968-5

Lagos-Cabré, R., Brenet, M., Díaz, J., Pérez, R. D., Pérez, L. A., Herrera-Molina, R., et al. (2018). Intracellular Ca2+ increases and Connexin 43 hemichannel opening are necessary but not sufficient for Thy-1-induced astrocyte migration. *Int. J. Mol. Sci.* 19, 2179–2195. doi: 10.3390/ijms19082179

Leyton, L., and Hagood, J. S. (2014). Thy-1 modulates neurological cell-cell and cell-matrix interactions through multiple molecular interactions. *Adv. Neurobiol.* 8, 3–20. doi: 10.1007/978-1-4614-8090-7_1

Leyton, L., Quest, A. F. G., and Bron, C. (1999). Thy-1/CD3 coengagement promotes TCR signaling and enhances particularly tyrosine phosphorylation of the raft molecule LAT. *Mol. Immunol.* 36, 755–768. doi: 10.1016/s0161-5890(99)00086-3

Leyton, L., Schneider, P., Labra, C. V., Rüegg, C., Hetz, C. A., Quest, A. F. G., et al. (2001). Thy-1 binds to integrin β3 on astrocytes and triggers formation of focal contact sites. *Curr. Biol.* 11, 1028–1038. doi: 10.1016/s0960-9822(01)break00262-7

Linden, R. (2017). The biological function of the prion protein: a cell surface scaffold of signaling modules. *Front. Mol. Neurosci.* 10:77. doi: 10.3389/fnmol.2017.00077

Liu, X., Wong, S. S., Taype, C. A., Kim, J., Shentu, T. P., Espinoza, C. R., et al. (2017). Thy-1 interaction with Fas in lipid rafts regulates fibroblast apoptosis and lung injury resolution. *Lab. Investig.* 97, 256–267. doi: 10.1038/labinvest.2016.145

Maldonado, H., Calderon, C., Burgos-Bravo, F., Kobler, O., Zuschratter, W., Ramirez, O., et al. (2017). Astrocyte-to-neuron communication through integrin-engaged Thy-1/CBP/Csk/Src complex triggers neurite retraction via the RhoA/ROCK pathway. *Biochim. Biophys. Acta* 1864, 243–254. doi: 10.1016/j.bbamcr.2016.11.006

Mizoguchi, F., Slowikowski, K., Wei, K., Marshall, J. L., Rao, D. A., Chang, S. K., et al. (2018). Functionally distinct disease-associated fibroblast subsets in rheumatoid arthritis. *Nat. Commun.* 9:789. doi: 10.1038/s41467-018-02892-y

Morgan, M. R., Humphries, M. J., and Bass, M. D. (2007). Synergistic control of cell adhesion by integrins and syndecans. *Nat. Rev. Mol. Cell Biol.* 8, 957–969. doi: 10.1038/nrm2289

Morris, R. J. (2018). Thy-1, a pathfinder protein for the post-genomic era. *Front. Cell Dev. Biol* 6:173. doi: 10.3389/fcell.2018.00173

Morris, R. J., Jen, A., and Warley, A. (2011). Isolation of nano-meso scale detergent resistant membrane that has properties expected of lipid "rafts.". *J. Neurochem.* 116, 671–677. doi: 10.1111/j.1471-4159.2010.07076.x

Nazari, B., Rice, L. M., Stifano, G., Barron, A. M. S., Wang, Y. M., Korndorf, T., et al. (2016). Altered dermal fibroblasts in systemic sclerosis display podoplanin and CD90. *Am. J. Pathol.* 186, 2650–2664. doi: 10.1016/j.ajpath.2016.06.020

Pan, C. Q., Sudol, M., Sheetz, M., and Low, B. C. (2012). Modularity and functional plasticity of scaffold proteins as p(l)acemakers in cell signaling. *Cell. Signal.* 24, 2143–2165. doi: 10.1016/j.cellsig.2012.06.002

Park, T. I. H., Feisst, V., Brooks, A. E. S., Rustenhoven, J., Monzo, H. J., Feng, S. X., et al. (2016). Cultured pericytes from human brain show phenotypic and functional differences associated with differential CD90 expression. *Sci. Rep.* 6:26587. doi: 10.1038/srep26587

Partida, G. J., Stradleigh, T. W., Ogata, G., Godzdanker, I., and Ishida, A. T. (2012). Thy-1 associates with the cation channel subunit HCN4 in adult rat retina. *Retin. Cell Biol.* 53, 1696–1703. doi: 10.1167/iovs.11-9307

Picke, A. K., Campbell, G. M., Schmidt, F. N., Busse, B., Rauner, M., Simon, J. C., et al. (2018b). Thy-1 deficiency augments bone loss in obesity by affecting bone formation and resorption. *Front. Cell Dev. Biol.* 6:127. doi: 10.3389/fcell.2018.00127

Picke, A. K., Campbell, G. M., Blüher, M., Krügel, U., Schmidt, F. N., Tsourdi, E., et al. (2018a). Thy-1 (CD90) promotes bone formation and protects against obesity. *Sci. Transl. Med.* 10:453. doi: 10.1126/scitranslmed.aao6806

Rege, T. A., and Hagood, J. S. (2006). Thy-1, a versatile modulator of signaling affecting cellular adhesion, proliferation, survival, and cytokine/growth factor responses. *Biochim. Biophys. Acta* 1763, 991–999. doi: 10.1016/j.bbamcr.2006.08.008

Russell, K. A., Chow, N. H. C., Dukoff, D., Gibson, T. W. G., La Marre, J., Betts, D. H., et al. (2016). Characterization and immunomodulatory effects of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells. *PLoS One* 11:e0167442. doi: 10.1371/journal.pone.0167442

Saalbach, A., Arnhold, J., Leßig, J., Simon, J. C., and Anderegg, U. (2008). Human Thy-1 induces secretion of matrix metalloproteinase-9 and CXCL8 from human neutrophils. *Eur. J. Immunol.* 38, 1391–1403. doi: 10.1002/eji.200737901

Sauzay, C., Voutetakis, K., Chatziioannou, A., Chevet, E., and Avril, T. (2019). CD90/Thy-1, a cancer-associated cell surface signaling molecule. *Front. Cell Dev. Biol.* 7:66. doi: 10.3389/fcell.2019.00066

Schmidt, M., Gutknecht, D., Simon, J. C., Schulz, J., Eckes, B., and Anderegg, U. (2015). Controlling the balance of fibroblast proliferation and differentiation: impact of Thy-1. *J. Invest. Dermatol.* 135, 1893–1902. doi: 10.1038/jid.2015.86

Shiga, K., Hara, M., Nagasaki, T., Sato, T., Takahashi, H., and Takeyama, H. (2015). Cancer-associated fibroblasts: their characteristics and their roles in tumor growth. *Cancers* 7, 2443–2458. doi: 10.3390/cancers7040902

Stacey, M., Chang, G. W., Davies, J. Q., Kwakkenbos, M. J., Sanderson, R. D., Hamann, J., et al. (2003). The epidermal growth factor-like domains of the human EMR2 receptor mediate cell attachment through chondroitin sulfate glycosaminoglycans. *Blood* 102, 2916–2924. doi: 10.1182/blood-2002-11-3540

Tjong, W. Y., and Lin, H. H. (2019). The RGD motif is involved in CD97/ADGRE5-promoted cell adhesion and viability of HT1080 cells. *Sci. Rep.* 9:1517. doi: 10.1038/s41598-018-38045-w

Van Zundert, B., Izaurieta, P., Fritz, E., and Alvarez, F. J. (2012). Early pathogenesis in the adult-onset neurodegenerative disease amyotrophic lateral sclerosis. *J. Cell. Biochem.* 113, 3301–3312. doi: 10.1002/jcb.24234

Wandel, E., Saalbach, A., Sittig, D., Gebhardt, C., and Aust, G. (2012). Thy-1 (CD90) Is an interacting partner for CD97 on activated endothelial cells. *J. Immunol.* 188, 1442–1450. doi: 10.4049/jimmunol.1003944

Wang, T., Ward, Y., Tian, L., Lake, R., Guedez, L., Stetler-Stevenson, W. G., et al. (2005). CD97, an adhesion receptor on inflammatory cells, stimulates angiogenesis through binding integrin counterreceptors on endothelial cells. *Blood* 105, 2836–2844. doi: 10.1182/blood-2004-07-2878

Ward, Y., Lake, R., Faraji, F., Sperger, J., Martin, P., Gilliard, C., et al. (2018). Platelets promote metastasis via binding tumor CD97 leading to bidirectional signaling that coordinates transendothelial migration. *Cell Rep.* 23, 808–822. doi: 10.1016/j.celrep.2018.03.092

Wen, H. C., Kao, C., Hsu, R. C., Huo, Y. N., Ting, P. C., Chen, L. C., et al. (2013). Thy-1-induced migration inhibition in vascular endothelial cells through reducing the RhoA activity. *PLoS One* 8:e61506. doi: 10.1371/journal.pone.0061506

Wetzel, A., Wetzig, T., Haustein, U. F., Sticherling, M., Anderegg, U., Simon, J. C., et al. (2006). Increased neutrophil adherence in psoriasis: role of the human endothelial cell receptor Thy-1 (CD90). *J. Invest. Dermatol.* 126, 441–452. doi: 10.1038/sj.jid.5700072

Zaidel-Bar, R., and Geiger, B. (2010). The switchable integrin adhesome. *J. Cell Sci.* 123, 1385–1388. doi: 10.1242/jcs.066183

Zaidel-Bar, R., Itzkovitz, S., Maandaposayan, A., Iyengar, R., and Geiger, B. (2007). Functional atlas of the integrin adhesome. *Nat. Cell Biol.* 9, 858–867. doi: 10.1038/ncb0807-858

Zhang, R., Liu, Y., Yan, K., Chen, L., Chen, X. R., Li, P., et al. (2013). Anti-inflammatory and immunomodulatory mechanisms of mesenchymal stem cell transplantation in experimental traumatic brain injury. *J. Neuroinflammation* 10:106. doi: 10.1186/1742-2094-10-106

Zhang, W., Trible, R. P., and Samelson, L. E. (1998). LAT palmitoylation: Its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity* 9, 239–246.

Zhou, Y., Hagood, J. S., Lu, B., Merryman, W. D., and Murphy-ullrich, J. E. (2010). Thy-1-Integrin αvβ5 interactions inhibit lung fibroblast contraction-induced latent transforming growth factor- beta1 activation and myofibroblast differentiation. *J. Biol. Chem.* 285, 22382–22393. doi: 10.1074/jbc.M110.126227

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Neuronal Signaling by Thy-1 in Nanodomains With Specific Ganglioside Composition: Shall We Open the Door to a New Complexity?

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Thy-1 is a small membrane glycoprotein and member of the immunoglobulin superfamily of cell adhesion molecules. It is abundantly expressed in many cell types including neurons and is anchored to the outer membrane leaflet *via* a glycosyl phosphatidylinositol tail. Thy-1 displays a number of interesting properties such as fast lateral diffusion, which allows it to get in and out of membrane nanodomains with different lipid composition. Thy-1 displays a broad expression in different cell types and plays confirmed roles in cell development, adhesion and differentiation. Here, we explored the functions of Thy-1 in neuronal signaling, initiated by extracellular binding of $\alpha_V\beta_3$ integrin, may strongly dependent on the lipid content of the cell membrane. Also, we assort literature suggesting the association of Thy-1 with specific components of lipid rafts such as sialic acid containing glycosphingolipids, called gangliosides. Furthermore, we argue that Thy-1 positioning in nanodomains may be influenced by gangliosides. We propose that the traditional conception of Thy-1 localization in rafts should be reconsidered and evaluated in detail based on the potential diversity of neuronal nanodomains.

Keywords: Thy-1, ganglioside, nanodomain, lipid rafts, neuronal signaling

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INTRODUCTION

Thy-1 is a small (17–18 kDa), *N*-glycosylated glycosylphosphatidylinositol (GPI)-anchored protein positioned in outer membrane leaflet domains enriched with cholesterol and gangliosides, called lipid rafts [molecular features, expression patterns and cell functions of Thy-1 are reviewed in Herrera-Molina et al. (2013) and in Leyton and Hagood (2014)]. Thy-1 is expressed in several cell types including human thymocytes, hematopoietic stem cells, glioblastoma cells, mesothelium precursor cells, neurons, and some subsets of fibroblasts among others. Depending on the cell type, the functions of Thy-1 include cell development and differentiation as well as regulation of adhesion and morphological changes in the context of cell-cell and cell-matrix contact (Leyton and Hagood, 2014).

The functions of Thy-1 are proposed to be regulated by the binding of endogenous ligands of which certain integrins are the most prominent ones (Herrera-Molina et al., 2013; Leyton and Hagood, 2014). The first ever characterized Thy-1-Integrin interaction is the one involving

extracellular binding of astroglial $\alpha_V\beta_3$ integrin and changes in the lateral diffusion as well as the nanoclustering state of Thy-1 in the neuronal membrane (Leyton et al., 2001; Maldonado et al., 2017). Notably, the engagement of $\alpha_V\beta_3$ integrin not only results in profound morphological changes and increased migration in the astrocytes (Avalos et al., 2004, 2009; Hermosilla et al., 2008; Henriquez et al., 2011), but also triggers Thy-1-depending intracellular signaling in neurons (Herrera-Molina et al., 2012, 2013). The $\alpha_V\beta_3$ integrin-triggered Thy-1 clustering has recently been shown to regulate inactivation and exclusion of the non-receptor tyrosine kinase Src from a Thy-1/C-terminal Src kinase (Csk)-binding protein (CBP)/Csk complex, resulting in p190Rho GTPase activation, cofilin and myosin light chain II phosphorylation, and consequently neurite shortening (Maldonado et al., 2017). However, it remains unknown whether these mechanisms initiated by $\alpha_V\beta_3$ integrin binding to Thy-1 are occurring in lipid rafts. Interestingly, super-resolution-suited fluorescent analogs of GPI-anchored proteins and gangliosides have recently been developed, expanding the toolbox to evaluate the interactions between these raft-associated molecules (Komura et al., 2016; Suzuki et al., 2017). In particular, these new studies have revealed gangliosides as highly dynamic components of rafts able to interact and regulate positioning of GPI-anchored proteins. Here, we briefly review literature demonstrating that Thy-1 is present in lipid rafts and that, in response to extracellular engagement, its mobility decreases in particular subsets of them. Also, we explore evidence showing that interactions between Thy-1 and raft-associated signaling intermediates occur in a delicate equilibrium within a nanoscale and millisecond time range. Finally, we hypothesize that correct Thy-1 signaling depends on the presence of an adequate lipid milieu and that, particular classes of gangliosides could be important for correct positioning and/or signaling functions of Thy-1 in rafts in the plasma membrane of neurons.

Thy-1-CONTAINING LIPID RAFTS: A TECHNICAL AND CONCEPTUAL OVERVIEW

Since Simons and Ikonen postulated the existence of functional lipid rafts (Simons and Ikonen, 1997), this area has been extensively studied in order to clarify the characteristics, composition, and functional role of lipid rafts in living cell systems. The original concept of how lipid rafts are organized, which should be acknowledged, has been subjected to revision and drastically changed over the years. Early experiments almost exclusively used cold detergent to extract these membrane domains and thus they were often accepted to be detergent-insoluble plasma membrane domains (Brown and Rose, 1992). Conceptually, they were thought to be patches of differently organized lipids that house specific transmembrane proteins. Later, additional research evolved the concept of lipid rafts from being stable and long-lived membrane patches to fluid and dynamic arrangements of clustered lipids and proteins (Owen et al., 2012). Although many studies have dwelled on whether lipid rafts even exist, it has become clear that lipid rafts exist and they may occupy only a fractional area of the plasma membrane. More recently, the use of super-resolution microscopy techniques applied to live-cell imaging has revealed rafts as actively changing and dynamically reorganizing nanodomains formed by different lipid and protein composition (reviewed in Sezgin, 2017).

Commonly used procedures for the characterization of lipid rafts are biochemical isolation methods based on ultracentrifugation in sucrose gradients and classical immuno-histochemical protocols (Pike, 2009; Williamson et al., 2010; Aureli et al., 2016). The results derived from these studies vary depending on used detergents, temperature, saline composition of buffers, etc. The choice of detergents is the most critical issue when the goal is to study native lipids, for example cholesterol organization or presence of gangliosides in rafts (reviewed in Klotzsch and Schutz, 2013). As today we know, biochemical isolation of rafts using different non-ionic detergents, namely Triton X-100, can produce a number of artifacts, including non-physiological clustering of certain lipids and proteins. Therefore, classically accepted results obtained using this detergent should be reconsidered and critically subjected to a new scrutiny. A possible way out of this problem could be the introduction of other detergents found to be less disruptive to the plasma membrane and more in tune with the composition and solubility properties of lipid rafts (Chamberlain, 2004; Heffer-Lauc et al., 2005, 2007; Williamson et al., 2010; Sonnino and Prinetti, 2013) (see later).

In contrast to the impossibility of fixing gangliosides, fixation procedures typically with *p*-formaldehyde (PFA) keep proteins in the membrane in immuno-histochemical studies. Clear-cut immuno-histochemical experiments concluded that the inclusion of 1% Triton X-100 (similar concentration is used in most raft isolation protocols) in blocking and primary antibody solutions caused a mild redistribution of Thy-1 from PFA-fixed wild-type to Thy-1 KO brain sections slices when they were incubated together in the same well. This is possibly due to extraction of Thy-1 and incorporation of its lipophilic GPI-anchor in detergent micelles as PFA does not completely fix GPI-anchored proteins (Tanaka et al., 2010). Despite the extractive capacity of the detergent, the immunoreactivity of remaining Thy-1 in different wild-type brain areas was grossly preserved after detergent exposure, indicating that most Thy-1 was fixed and resistant to extraction (Heffer-Lauc et al., 2005, 2007). In PFA-fixed neuronal cultures, Thy-1 staining on the cell surface is very well preserved after the use of Triton X-100-enriched solutions (Herrera-Molina et al., 2012, 2013; Maldonado et al., 2017). These studies showed that Thy-1 (just as any other transmembrane protein) resists detergent-mediated extraction most likely thanks to the fixative-induced cross-linking with other membrane proteins in intimate contact within the lipid raft. Nevertheless, PFA-promoted protein crosslinking is by itself an inevitable pitfall which should be controlled and/or complemented by alternative staining procedures. In neuronal cultures for example, live cell staining with monoclonal antibodies and super-resolution microscopy have been used to confirm changes in Thy-1 clustering (Herrera-Molina et al., 2013; Maldonado et al., 2017). Alternatively, new fixatives have been characterized specially for the use of super-resolution microscopy (Richter et al., 2018).

For years, the inability to isolate rafts at physiological temperature prolonged the debate on the existence of these nanodomains in living cells (London and Brown, 2000; Lingwood and Simons, 2007). Temperature and ion concentration have been proven to influence lipid raft isolation. Chen X. et al. (2009), a publication from Morris's lab, proposed that the problem of obtaining "physiological rafts" is a technical one caused by the disruption of the inner layer of the plasma membrane when in contact with detergents, such as Triton X-100 dissolved in buffers with inappropriate cation composition (Pike et al., 2002; Schuck et al., 2003; Koumanov et al., 2005). To solve this problem, the authors introduced detergent–containing buffers to mimic the intracellular ionic environment which prevented the disruption of the inner layer of the plasma membrane obtained from rodent brains. In addition to the provided biochemical evidence, the stabilization of membrane domains during isolation at 37°C was demonstrated by obtaining small nano-meso scale rafts of < 100 nm in size, as shown using immune-gold labeled antibodies and electron microscopy (Chen X. et al., 2009; Morris et al., 2011). Furthermore, they showed that the use of the new buffer formulation in combination with the detergents Brij98 or Brij96 further optimizes the isolation of brain rafts at physiological temperature (Chen X. et al., 2009; Morris et al., 2011).

Thy-1 is present in domains enriched with fully saturated lipids, which are distinguishable from prion protein PrP-containing rafts with significantly more unsaturated and longer chain lipids (Brugger et al., 2004). Confirming these results, the existence of independent Thy-1- or PrP-containing domains has been observed in brain membrane preparations with preserved inside-out orientation and isolated at physiological temperature (Chen X. et al., 2009; Morris et al., 2011). These observations strongly support the existence of different lipid raft populations, which are easily distinguishable in their composition. Moreover, it has been shown that Thy-1-containing, but not PrP-containing, lipid nanodomains are associated with actin, strengthening the idea of a tight interaction between Thy-1 and cytoskeletal/cytoplasmic components (Chen X. et al., 2009; Morris et al., 2011). Therefore, biochemical isolations of lipid rafts have not only provided the basis for the gross understanding of the differences in protein composition, but have also given functional meaning to subclasses of lipidic nanodomains. From this literature (and other), it is clear that GPI-anchored proteins like Thy-1, transmembrane proteins, intracellular signaling intermediates, and a variety of lipids may undergo interdependent interactions to form an undetermined number of different types of rafts.

Mobility and Nanoclustering of Thy-1 in Lipid Rafts

Biochemical assessments to characterize the presence of Thy-1 in certain rafts have been complemented with high-resolution imaging techniques aiming to observe the localization and behavior of the molecule inside and outside of rafts. More than 25 years ago, classical biochemical experimentation and liquid-phase chromatography revealed that Thy-1 forms multimers of 45–50 and 150 kDa in primary neurons and neuron-like PC12 cells (Mahanthappa and Patterson, 1992). Also, a number of reports have used electron microscopy-associated immunogold particles to describe the spontaneous formation of highly compact nanoclusters as small as 20–100 nm, comprising 2–20 molecules of Thy-1 (Brugger et al., 2004; Chen X. et al., 2009; Morris et al., 2011). More recently, it has been demonstrated that cholesterol in the outer leaflet of the plasma membrane allows tight contact between GPI-anchored proteins like Thy-1, CD59, and even GPI-anchored Green Fluorescent Protein, as these molecules have been observed as close as 4-nm apart using homo-FRET or single molecule tracking (SMT) (Sharma et al., 2004; Chen et al., 2006; Chen Y. et al., 2009; Komura et al., 2016; Suzuki et al., 2017). Thus, it has been proposed that cholesterol-associated nanoclusters of these GPI-anchored proteins may be functional units linked to protein complex formation to regulate signal transduction. This idea is supported by accumulated evidence indicating that the miscibility of lipid components in the plasma membrane may allow the coupling of the outer leaflet with the inner leaflet of the bilayer, facilitating the communication of two proteins on opposite sides of the membrane (Kusumi et al., 2004, 2010; Chen et al., 2006; Chen Y. et al., 2009; Suzuki et al., 2007a,b). Considering this scenario, the coincidental clustering of a critical number of Thy-1 molecules with an environment of saturated lipids in the external layer would act as a trigger for the reorganization of inner leaflet rafts.

Rafts are formed by the lateral assembly of cholesterol, phosphatidylcholine, and sphingolipids like gangliosides in the outer layer of cell membranes (Simons and Ikonen, 1997; Quest et al., 2004). Indeed, cholesterol – despite its rigid and bulky tetracyclic structure - is an essential component as it interacts with other lipids to form 5–200 nm patches with limited stability in the time range of milliseconds to minutes (Kusumi et al., 2004, 2010; Honigmann et al., 2014). From this, it is believed that the proper organization and lipid content in rafts can provide the correct environment for the functioning of more than 250 identified transmembrane and GPI-anchored raft-associated proteins in cell membranes from different sources (Santos and Preta, 2018). The plethora of lipid-protein interactions most likely defines the versatility, stability and specific functionality of these microdomains (Skibbens et al., 1989; Sargiacomo et al., 1993; Danielsen and van Deurs, 1995; Dietrich et al., 2001; Silvius, 2003; Hanzal-Bayer and Hancock, 2007). As an example, both assembly and disassembly of lipid rafts facilitates the effective activation of locally concentrated receptors by extracellular ligands as well as the posterior interaction with downstream effectors, adding speed and specificity to the ligand-receptor-encoded initiation of cell signaling (Pereira and Chao, 2007; Suzuki et al., 2007a,b; Lingwood and Simons, 2010; Pryor et al., 2012). Supporting the dynamism of rafts in terms of heterogeneity and short lifetimes, the use of stimulated emission depletion (STED), SMT, foster resonance energy transfer (FRET), and other super-resolution imaging techniques has helped to visualize protein–protein, protein–lipid, and lipid–lipid interactions becoming transiently stabilized and then disassembled in intact plasma membranes (Honigmann et al., 2014).

Changes in the aggregation state of Thy-1 and other GPI-anchored proteins induced by extracellular engagement have been observed using fast and super-resolution imaging techniques. Additionally, both important technical and conceptual advances have been made in the understanding of the physical dimensions ruling the lateral mobility and clustering of GPI-anchored proteins in cholesterol rafts (Kusumi et al., 2004, 2010; Hell, 2007; Honigmann et al., 2014). Using SMT with a 33-ms resolution, Kusumi's lab has shown that incubation with antibody-coated 40-nm gold particles clusters 3–9 CD59 molecules, which is enough to promote alternating periods of actin dependent temporary immobilization of the molecules with lifetimes of 200-ms up to 8-s (exponential lifetime = 100-ms) in epithelial and fibroblastic cell lines. The arrested CD59 molecules remained in a compartment of 110-nm in diameter (conventional resolution of fluorescent microscopes 250–400-nm), indicating that immobilization of CD59 is accompanied by limited diffusion in nano-rafts (Suzuki et al., 2007a,b). Jakobson's lab, also using fibroblasts, SMT with a 33-ms resolution, and antibody-coated 40-nm gold particles, described that the clustering of Thy-1 induces immobilization of the molecule during a slightly broader time rage of 300-ms up to 10-s (Chen et al., 2006). Moreover, both labs demonstrated that the arrest and positioning of the Thy-1 and CD59 clusters in lipid rafts strongly depend on cholesterol integrity. Therefore, it is clear that ultra-fast, but also slow transient arrests of GPI-anchored proteins are triggered by extracellular engagement in cholesterol rafts. Nevertheless, the results obtained using these artificial ligands to promote clustering of the GPI-anchored proteins, like Thy-1, could not fully describe the natural responses to endogenous ligands to a necessary degree.

EXTRACELLULAR BINDING OF GLIAL $\alpha_V\beta_3$ INTEGRIN CONFINES NEURONAL Thy-1

For decades, an endogenous ligand for Thy-1 remained in the dark. In 2001, $\alpha_V\beta_3$ integrin expressed by astrocytes was identified as a receptor for Thy-1 (Leyton et al., 2001). Leyton's lab has characterized in detail the direct binding between $\alpha_V\beta_3$ integrin and the RGD-like sequence (RLD, positions 35–37 accession number AAA61180.1) of Thy-1 by surface plasmon resonance (Choi et al., 2005; Hermosilla et al., 2008), confocal microscopy (Herrera-Molina et al., 2012), and recently using molecular force spectroscopy (optical tweezers) (Burgos-Bravo et al., 2018). Importantly, the same lab has revealed crucial $\alpha_V\beta_3$ integrin-dependent and Thy-1-induced signaling events, promoting morphological changes in astroglial cells (Avalos et al., 2004, 2009; Hermosilla et al., 2008; Henriquez et al., 2011; Alvarez et al., 2016; Lagos-Cabré et al., 2017, 2018; Burgos-Bravo et al., 2018). Supporting a paradigm of bidirectional communication between neurons and astrocytes, the astroglial $\alpha_V\beta_3$ integrin was found to also act as a ligand for neuronal Thy-1 to trigger signaling events and retraction of axons and dendrites in neurons (Herrera-Molina et al., 2012, 2013; Maldonado et al., 2017).

The binding of $\alpha_V\beta_3$ integrin promotes Thy-1 clustering on the neuronal cell surface (Herrera-Molina et al., 2012, 2013; Maldonado et al., 2017). Using super resolution STED microscopy followed by image deconvolution procedures (lateral resolution of 40 nm), single Thy-1 nanoclusters were found as small as 90 nm in diameter (Herrera-Molina et al., 2013; Maldonado et al., 2017). Upon extracellular binding of $\alpha_V\beta_3$ integrin, Thy-1 clusters with a diameter of 300–400 nm were detected abundantly with extensive aggregation (**Figure 1** and Maldonado et al., 2017). Although unitary lifetimes of $\alpha_V\beta_3$ integrin-bound Thy-1 clusters have not been evaluated yet, a highly dynamic process is expected. Indeed, a single application of $\alpha_V\beta_3$ integrin was sufficient to reduce the average velocity and displacement area of quantum dot (QD)-labeled Thy-1 molecules, pointing to diminished lateral mobility of $\alpha_V\beta_3$ integrin-bound Thy-1 clusters (Maldonado et al., 2017). Interestingly, one fraction of Thy-1 molecules (∼60%) was fast ($\geq$5-μm/s), whereas the other one (40%) was comparatively slow ($\leq$5-μm/s) in control neurons. After binding of $\alpha_V\beta_3$ integrin, a smaller fraction of Thy-1 molecules remained fast (40%) (**Figure 1** and Maldonado et al., 2017). Additionally, as $\alpha_V\beta_3$ integrin binding reduced the mean square displacement (MSD) of Thy-1 molecules (**Figure 1**), it is possible to speculate that a specific fraction of Thy-1 molecules (20%) is sensitive to the interaction with $\alpha_V\beta_3$ integrin in neurons. Also, considering the high degree of subcellular compartmentalization of neurons, it is tempting to propose the existence of different subclasses of Thy-1 clusters in dendrites, axons, and/or synapses attending functional specializations of each of these cell compartments.

The evidence points toward a mechanism whereby the clustering of Thy-1 initiates intracellular downstream signals through the single-pass transmembrane adaptor protein CBP (C-terminal Src kinase binding protein). CBP is palmitoylated allowing localization in rafts (Brdicka et al., 1998; Zhang et al., 1998; Chen Y. et al., 2009). CBP plays an obligatory role in the transient arrest of Thy-1 molecules in rafts (Chen Y. et al., 2009) and contains intracellular tyrosine phosphorylation residues that serve as docking sites for Src family kinase (SFK) proteins, including Src and Csk (Wong et al., 2005; Solheim et al., 2008). Both clustering and immobilization of Thy-1 in rafts require SFK activity as demonstrated using QD-associated SMT in fibroblasts (Chen Y. et al., 2009). Moreover, antibody-induced Thy-1 clustering leads to recruitment of SFK to the membrane and modulates the activity of these kinases in a number of experimental settings (Barker et al., 2004; Chen et al., 2006; Yang et al., 2008). In neurons treated with $\alpha_V\beta_3$ integrin, about 15–20% of Thy-1 nanoclusters have been found to co-localize with CBP as determined using two-channel STED microscopy (Maldonado et al., 2017). Under the same experimental conditions, more CBP co-localized with Csk, which is known to phosphorylate Src at Tyr527 (Chen Y. et al., 2009; Lindquist et al., 2011). Therefore, it was concluded that the binding of $\alpha_V\beta_3$ integrin to Thy-1 increases the co-localization of clusters of Thy-1, CBP, and Csk in the cell membrane of neurons. Nevertheless, the lipidic nano-environment in which clustering of Thy-1-CBP-Csk took place remains unknown.

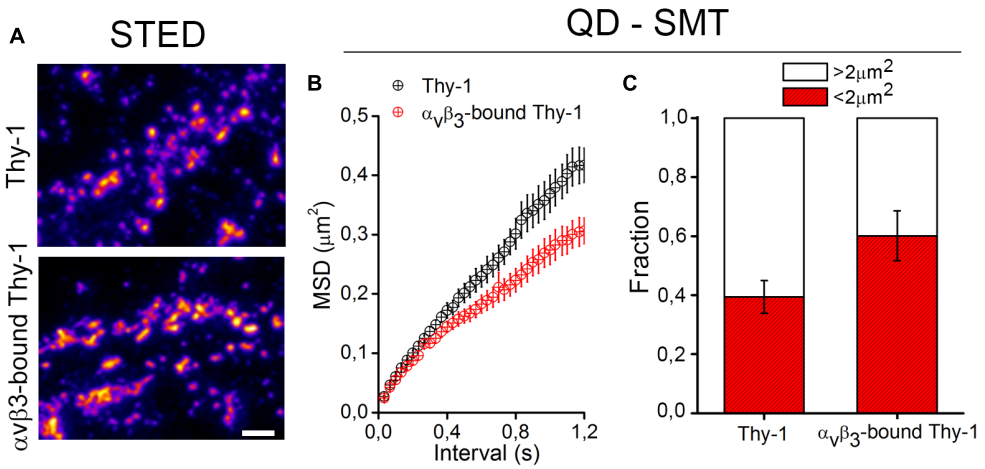

FIGURE 1 | Changes in clustering and confinement of Thy-1 induced upon $\alpha_v\beta_3$ integrin binding in the neuronal membrane. **(A)** As shown in Maldonado et al. (2017), cultured rat neurons were treated with a soluble form of the $\alpha_v\beta_3$ integrin, fixed with 4% PFA for 8 min, and stained with a mouse monoclonal anti-Thy-1 antibody (clone OX-7) followed by Atto647N-conjugated secondary antibodies. Then, Thy-1 nanoclusters were visualized using a super-resolution stimulated emission depletion (STED) microscope. **(B,C)** Single molecule tracking (SMT) of Thy-1 molecules attached to quantum dots (QD) is described in Maldonado et al. (2017). Further analysis of mean square displacement (MSD) **(B)** and the total fraction of molecules moving in areas with 2 μm^2 or more **(C)** confirmed that binding of $\alpha_v\beta_3$ integrin decreases the lateral mobility and increases the confinement of Thy-1.

BRIEF OVERVIEW ON NEURONAL GANGLIOSIDES

Gangliosides are sialic acid containing glycosphingolipids, abundantly present in the outer leaflet of the plasma membrane of all cell types (for detailed review of gangliosides see Schnaar et al., 2014). Gangliosides are synthesized in a stepwise manner by sequential addition of monosaccharides on a lipid backbone of ceramide *via* glycosyltransferase activities of different specificity to form oligosaccharide chain. The addition of sialic acid on specific positions in the oligosaccharide chain defines different ganglioside series. Due to their large number and overpowering complexity, gangliosides are still classified by Svennerholm's nomenclature into groups a, b, and c, depending on the number of sialic acids bound to the internal galactose, and the asialo-group if they have no sialic acid bound to the internal galactose (Svennerholm, 1963, 1980).

Functions of gangliosides include signal transduction, adhesion, cell recognition as well as positioning and function of proteins inside the plasma membrane of neurons (reviewed in detail in Schnaar et al., 2014). The importance of gangliosides for neuronal function has been demonstrated using mutant mice models with disrupted ganglioside synthesis and aberrant ganglioside composition (for example, *B4galnt1*-null mice lack GM2/GD2 synthase expression and thus the four most abundant brain gangliosides (GM1, GD1a, GD1b, and GT1b) are no longer produced). *B4galnt1*-null mice display normal total levels, production, and degradation of cholesterol as well as they do not present any difference in cholesterol turnover compared to wild-type mice (Li et al., 2008). Their phenotype includes axon degeneration, neuropathies, and deficits in reflexes, strength, coordination and posture. Also, male *B4galnt1*-null mice are infertile (Takamiya et al., 1996, 1998; Sheikh et al., 1999; Chiavegatto et al., 2000). At the molecular level, lateral interaction of gangliosides with proteins provides an additional level of regulation of neuronal signaling (Lopez and Schnaar, 2009; Prinetti et al., 2009). Studies have shown that gangliosides can modulate EGF and VEGF receptor sensitivity to their ligands (Bremer et al., 1986; Liu et al., 2006; Mukherjee et al., 2008). Furthermore, endogenous GM1 functions as a specific activator of Trk receptors and is capable of enhancing their activation in response to stimulation with NGF (Suzuki et al., 2004). This effect is most likely due to the enhancement of Trk-associated tyrosine kinase activity elicited by NGF (Mutoh et al., 1995). Therefore, it has been stated that gangliosides are essential regulators of normal neuronal function capable of tuning a number of signaling mechanisms (further argumentation is reviewed in Lopez and Schnaar, 2009; Schnaar et al., 2014).

Classical analyses of the expression and distribution of gangliosides have been based on their high extractability with different organic solvents (Svennerholm, 1963). After their extraction, gangliosides have been separated and analyzed using HPTLC (high performance thin layer chromatography) (**Figure 2**). Additionally, both structural characterization and quantification of the lipid content have been assessed using mass spectrometry (29). These methods have been useful to define the composition and abundance of gangliosides in different tissues and cell types. In the human brain, and very similarly in the rodent brain (**Figure 2**), GM1, GD1a, GD1b, and GT1b together sum up to 97% of the total ganglioside content. Ganglioside distribution has been studied in brain tissue using specific primary antibodies followed by 3-3′-Diaminobenzidine-based staining similarly as for Thy-1 (Heffer-Lauc et al., 2005, 2007) or other CAMs like Neuroplastin (Mlinac et al., 2012; Herrera-Molina et al., 2017; Ilic et al., 2019). However, special caution is required regarding the detergent used during the procedures

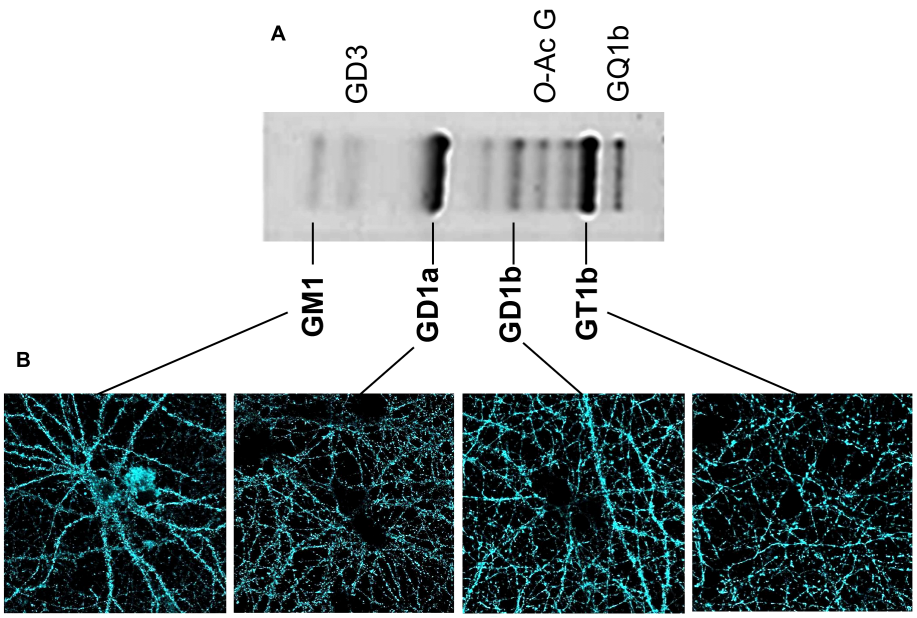

FIGURE 2 | Content of brain gangliosides and visualization of gangliosides in neuronal membrane. **(A)** Separation of the ganglioside types obtained from homogenates of hippocampal cell membranes was performed using HPTLC as described (Svennerholm, 1963). Briefly, gangliosides were extracted from homogenized tissue using a chloroform/methanol/water mix and then purified using a SPECTRA/POR 6 Dialysis Tubing membrane. After drying, samples were spotted on HPTLC plate developed in chloroform/methanol/CaCl2 mix. Gangliosides were detected with a resorcinol-HCl reagent. The identity of each ganglioside type is indicated. **(B)** Confocal microscopy and KO-controlled primary monoclonal antibodies (Schnaar et al., 2002) were used to evaluate independent presence of each of the gangliosides GM1, GD1a, GD1b, and GT1b on the cell surface of living hippocampal neurons. Our procedure to stain living neurons in the absence of fixative and detergents has been described (Herrera-Molina et al., 2012, 2014). Briefly, living rat neurons were directly treated with each of the KO-controlled anti-ganglioside monoclonal antibody diluted in culture media (1:500) for 20 min at 37°C, 5% CO_2. Then, neurons were carefully washed with culture media, fixed with PFA for 10 min at 37°C, stained with Alexa 488-conjugated secondary antibodies for 1 h, and mounted with Mowiol. All four gangliosides displayed a specific patched signal.

as inappropriate conditions produce artifacts as drastic as loss and re-distribution of several ganglioside types. As described by Ronald Schnaar's lab, the use of some bench detergents, including CHAPS, SDS, and Triton X-100 in PFA-fixed wild-type brain sections, results in a major extraction of gangliosides from their original location (Heffer-Lauc et al., 2005, 2007). The latter effect of detergents was so dramatic that a clear transfer of wild-type gangliosides to the white matter of brain slices of *B4galnt1*-null mice was observed. Authors have optioned to avoid any detergent in ganglioside staining of brain sections. These studies have shown that GM1 is normally concentrated in white matter tracts throughout the adult mice brain, whereas GD1a staining displays a complementary distribution in gray matter. GT1b and GD1b have been found in both gray and white matter (Heffer-Lauc et al., 2005, 2007; Vajn et al., 2013; Schnaar et al., 2014). Unfortunately, in these experimental conditions, uneven antibody diffusion cannot be completely ruled out limiting high-resolution imaging approaches. Lately, we have assessed the visualization and subcellular distribution of the four main brain gangliosides in neurons by combining KO-controlled monoclonal antibodies (Schnaar et al., 2002; **Supplementary Figure S1**) and high-resolution confocal microscopy. As mentioned before, gangliosides cannot be directly fixed using PFA and they are sensitive to detergent extraction. Therefore, as a first approach, we have used these specific monoclonal antibodies to perform live cell staining either at room temperature or 37°C. Then, one-to-one ganglioside-antibody complexes are fixed with PFA. No detergent is ever used throughout the procedures. Surprisingly, we have obtained a good staining of cell surface located gangliosides (**Figure 2**). Also, we have visualized the distribution of patches of GM1, GD1a, GD1b, and GT1b throughout soma, dendrites and axons (**Figure 2**). This promising and simple procedure will be applied to further study these distributions of gangliosides in combination with super-resolution STED microscopy in living neurons. It would be particularly interesting to study the distribution and composition of, what could be, nanodomains differentially enriched with particular gangliosides on the neuronal surface.

WHAT CAN GANGLIOSIDES TELL US ABOUT RAFTS?

Visualization of the nano-landscape of randomly scattered GM1 patches has been performed with near-field scanning optical microscopy (NSOM). This technique takes advantage of the evanescent field exiting a subwavelength excitation source, therefore being particularly suited for nanoscale optical imaging ($\geq$80 nm of lateral resolution) on intact biological membranes (van Zanten et al., 2010). In particular, organized

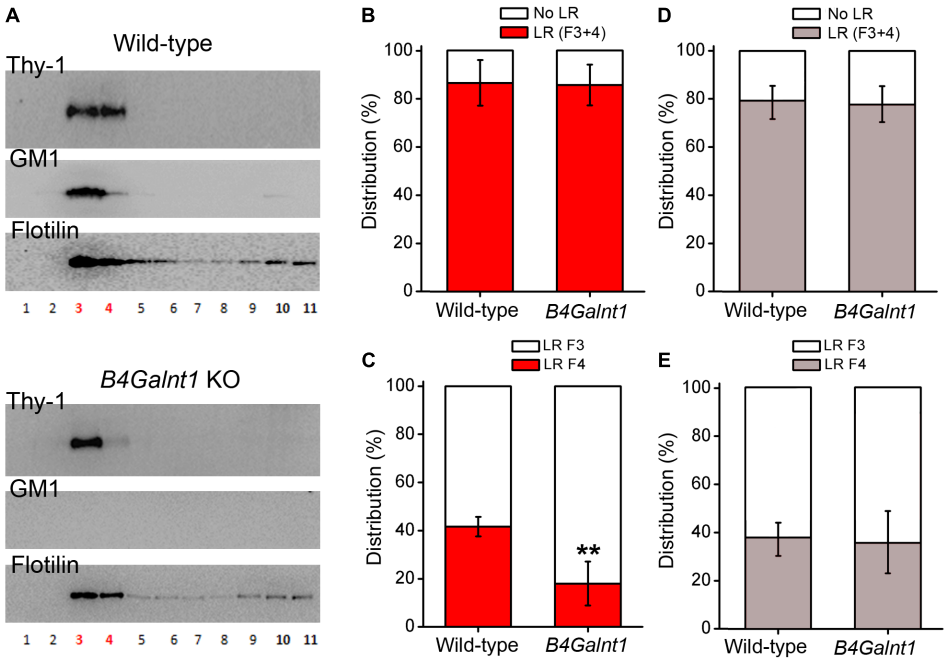

FIGURE 3 | Thy-1 distribution in sucrose density gradients of wild-type and *B4Galnt1-null* brain membranes. **(A)** Representative Western blots of sucrose gradient fractions obtained from total membrane homogenates of wild-type and *B4Galnt1* KO brain cortices as indicated. Lipid rafts isolation is based on published protocols (Persaud-Sawin et al., 2009; Hattersley et al., 2013) with some modifications. After homogenization, nuclear fraction was removed and cell membrane pellet was obtained by centrifugation (30 min, 100,000 × *g*). This pellet was further homogenized in a lysis buffer containing BrijO20 and ultracentrifuged at 140,000 × *g* in a discontinuous sucrose gradient (85% mixed with sample, 35 and 3%). Next day, all fractions were collected for analysis. *B4galnt1*-null mice lacking GM2/GD2 synthase have been previously characterized (Takamiya et al., 1996, 1998; Sheikh et al., 1999; Chiavegatto et al., 2000; Li et al., 2008) and they cannot synthesize any of the four most abundant brain gangliosides GM1, GD1a, GD1b, and GT1b. We confirmed complete absence of GM1 (this Figure) and GT1b (**Supplementary Figure S1**) in *B4Galnt1* KO brain material using cholera toxin and specific primary antibodies, respectively. Isolation of lipid raft fractions (3 and 4 in red color) was confirmed by detection of flotilin. **(B)** Quantification of accumulative distribution of Thy-1 in bulk membrane fractions (F10 + F11, no lipid rafts: No LR) and in lipid raft fractions (F3 + F4, lipid rafts: LR) of each genotype. **(C)** Quantification of the distribution of Thy-1 in each of the two lipid raft fractions. Distribution of Thy-1 between the raft fractions 3 and 4 seems modified due to the alteration of ganglioside content in *B4Galnt1*-null brain membranes (**$P < 0.01$ for fraction 4 comparing genotypes, Mann–Whitney test). **(D)** Quantification of accumulative distribution of flotilin was performed as for Thy-1 in **(B)**. **(E)** Distribution of flotilin between the raft fractions 3 and 4 was performed as for Thy-1 in **(C)**. Data are expressed as mean ± SD of 5 independent gradients.

GM1 nanodomains with a size < 120-nm, separated by an inter-nanodomain distance of approximately 300 nm, were found in the plasma membrane of fibroblasts. Furthermore, this nanodomain organization was not dependent on the temperature, but on the presence of cholesterol and an intact actin-based cytoskeleton (van Zanten et al., 2010). In other studies using antibodies conjugated to gold particles and electron microscopy, either ganglioside GM1 or GM3 were observed forming patches separately and only co-localizing with each other (GM1 and GM3 containing patches) in less than 15% of the cases on the cell surface of fibroblasts (Fujita et al., 2007). Therefore, although composition of lipid rafts can be very divers, their formation and localization seems to be organized throughout the plasma membrane.

Direct visualization of gangliosides and GPI-anchors in living cell membranes has been achieved using chemically synthetized fluorescent analogs and super-resolution STED microscopy (Eggeling et al., 2009; Polyakova et al., 2009; Komura et al., 2016; Suzuki et al., 2017). In 2009, Hell's lab demonstrated that both Atto647N-conjugated GPI-anchors and GM1 have similar diffusion properties and confinements in rafts (called "trapping"; Eggeling et al., 2009). Notably, the addition of cholesterol-depleting agents similarly reduced the trapping of GPI-anchors and GM1 (Eggeling et al., 2009). Despite recent criticism pointing to insufficient characterization of the fluorescent analogs (Komura et al., 2016; Suzuki et al., 2017), these studies suggest that GPI-anchored proteins and GM1 may share similar lateral diffusion properties in cholesterol rafts. Very recently, a new generation of super-resolution-suited fluorescent analogs has been developed to visualize the relationship between raft-associated GPI-anchored proteins and gangliosides. The authors claimed that the main strength of the new analogs is that they partition in rafts just as the endogenous molecules do (Komura et al., 2016; Suzuki et al., 2017). Furthermore, this method allowed direct observation of positioning and movement of ganglioside and GPI-anchored protein molecules as well as their co-localization without effects of crosslinking. When the raft structure was analyzed by single-molecule imaging, it was determined that ganglioside fluorescent analogs dynamically enter and leave rafts. Inside rafts, ganglioside analogs were immobile for approximately 100 ms, while outside the raft they were constantly moving. The arrest of the ganglioside

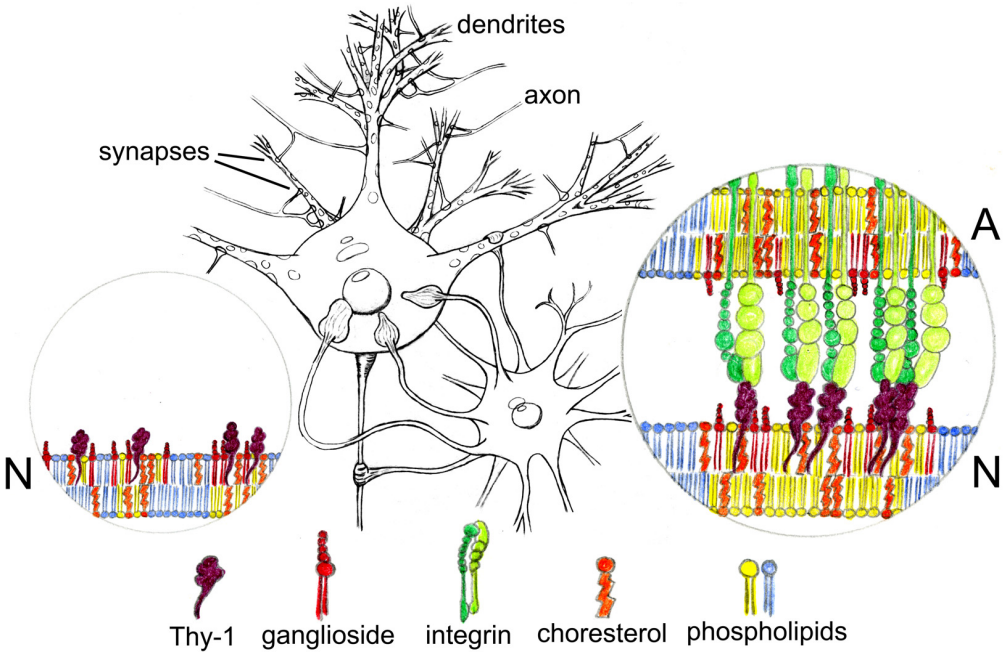

FIGURE 4 | Hypothetical participation of gangliosides in the nanoclustering of neuronal Thy-1 induced by astroglial $\alpha_v\beta_3$ integrin. Soma, axon, and dendrites of one neuron are contacted by end feet of one astrocyte (**middle drawing**). We propose gangliosides could be important for correct positioning and signaling functions of Thy-1 in rafts in the plasma membrane of neurons. If this turns out to be true, then, gangliosides should influence the diffusion and clustering properties of Thy-1 along the neuronal surface (**N, left circle**) as well as the clustering of Thy-1 molecules induced by $\alpha_v\beta_3$ integrin expressed by astrocytes (**A, right circle**). $\alpha_v\beta_3$ integrin-bound Thy-1 might be integrated in nanodomains with particular ganglioside composition to initiate signaling which may differently impact the functioning of each cell compartment.

analogs inside rafts was dependent on actin cytoskeleton and cholesterol integrity (Komura et al., 2016). Additionally, the authors have proposed that cholesterol rafts provide a nano-environment for different proteins, and that gangliosides may have regulatory effects on the recruitment of these proteins. Furthermore, gangliosides could also strengthen interactions between GPI-anchored proteins and other lipids in rafts (Komura et al., 2016; Suzuki et al., 2017).

DO GANGLIOSIDES INFLUENCE CLUSTERING/DISTRIBUTION OF Thy-1 IN NEURONS?

In eukaryotes, gangliosides assemble with other glycosphingolipids and cholesterol to form lipid rafts (Sonnino et al., 2007). It is known that depletion of cholesterol causes deficient clustering of GPI-anchored proteins, including Thy-1 and CD59 (Simons et al., 1999; Sharma et al., 2004; Chen et al., 2006; Chen Y. et al., 2009; Komura et al., 2016; Suzuki et al., 2017), and impairs lipid raft structure (Kabouridis et al., 2000; Buschiazzo et al., 2013). Although, gangliosides have been found to be permissive with the formation of GPI-yellow fluorescent protein clusters in living cell membranes (Crespo et al., 2002), neither deficient nor altered ganglioside content that leads to lipid raft disruption and/or impairs the clustering of GPI-anchored proteins have been studied in detail.

As mentioned, studies have suggested that gangliosides are important for positioning and clustering of GPI-anchored proteins in cholesterol rafts, rather than being necessary for the raw structuring of the raft itself (Eggeling et al., 2009; Komura et al., 2016; Suzuki et al., 2017). Indeed, authors have reported that positioning of Thy-1 within rafts depends on ganglioside composition as concluded after experiments using cerebellum membrane preparations from wild-type and double mutant mice lacking GM2/GD2 and GD3 synthases (Ohmi et al., 2009). In this study, most Thy-1 immunoreactivity drastically shifted from one to another raft fraction obtained by sucrose gradient centrifugation (Ohmi et al., 2009). Ohmi et al. (2009) concluded that the precise positioning of Thy-1 inside rafts seems to depend on gangliosides. In agreement with Ohmi et al. (2009) we have observed that Thy-1 is present in *B4galnt1*-null lipid rafts (lacking the four main brain gangliosides, see before and **Figure 2**), but shifted from raft fraction 4 to the lighter fraction 3 (**Figure 3**). Interestingly, the total content of Thy-1 in *B4galnt1*-null rafts (fraction 3 + 4) was not different to wild-type rafts (also fraction 3 + 4) (**Figure 3**). Considering that the total content of both cholesterol and sialic acid bound to simpler gangliosides do not differ between *B4galnt1*-null and wild-type mice (Li et al., 2008), our results suggest that altered ganglioside production impaired fine distribution of Thy-1 within *B4galnt1*-null rafts. Supporting this idea, incubation with exogenous GM1 directly and acutely added to the kidney cell line MDCK cells diminished the clustering of the GPI-anchored protein GH-DAF in rafts

(Simons et al., 1999). In constructed monolayers of synthetic lipid mixtures with defined lipid composition, the presence of Thy-1 in artificial rafts was found to be reduced when GM1 was added, most likely because GM1 and Thy-1 competed for positioning inside rafts (Dietrich et al., 2001). Thus, it is possible to speculate that exogenously added GM1 formed aggregates reducing Thy-1 mobility in raft-like domains (Marushchak et al., 2007). Although it will be also necessary to proof the potential influence of other lipids as cholesterol, additional available evidence supports the possibility that ganglioside milieu influences Thy-1 location in rafts. Indeed, the literature shows that the distribution of flotilin in raft fractions from neurons, brain tissue, myocites, and erytrocytes is strongly sensitive to cholesterol alterations (Samuel et al., 2001; Kokubo et al., 2003; Jia et al., 2006; Domingues et al., 2010; Sones et al., 2010). These studies consistently show that flotilin distribution reflects and/or reports cholesterol-dependent raft integrity. We shown that the distribution of flotilin is not changed in the *B4galnt1*-null raft fractions with altered ganglioside composition pointing to a rather specific change in raft composition rather than a general modification in the raft integrity (**Figure 3**).

CONCLUSION

For decades, the small GPI-anchored molecule Thy-1 had hidden its charms and remained an orphan in silence. For years, the discovery of Thy-1 as a raft-associated protein served to study these nanoscopic domains. Finally, the development and popularization of super-resolution microscopy techniques allowed to access Thy-1 properties such as lateral mobility, cluster formation, and partition features within the lipidic environment of the cell membrane. The characterization of an endogenous ligand for Thy-1, the $\alpha_V\beta_3$ integrin, made it possible to reveal detailed mechanisms involved in Thy-1-dependent *cis* signaling in neurons. As experiments show, intracellular signaling emanated from $\alpha_V\beta_3$ integrin-Thy-1 binding in neurons depends on the initial enrolling of the raft-associated transmembrane transducer CBP and Src kinase to regulate the stability of neuronal cytoskeleton. However, it is still a mystery whether these molecular events are actually occurring in neuronal rafts. The fine-tuning of protein-protein interactions in the outer layer of the cell membrane may be influenced by the lipid environment, in particular by cholesterol and gangliosides, which are two key components of rafts.

We propose that the correct ganglioside composition is necessary for distribution, clustering, and function of Thy-1 in neurons (**Figure 4**). The potential significance of this putative association could be reflected on the capacity of Thy-1 to initiate signaling mechanisms in rafts. In particular, this could be additionally tested by analyzing the $\alpha_V\beta_3$ integrin-Thy-1-dependent *cis* signaling events that occur at the plasma membrane (**Figure 4**; Herrera-Molina et al., 2013; Maldonado et al., 2017) in neuronal systems where ganglioside composition is, ideally, acutely modified. Finally, the pieces of the puzzle displayed are waiting to be gathered together into correct assembly.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

Experiments were carried out at Animal Facility of Croatian Institute for Brain Research and were approved by Croatian Ministry of Agriculture, under class number 602-04/14-08/06 and registration number 2158-61-07-14-118.

AUTHOR CONTRIBUTIONS

KI contributed the ganglioside isolation, lipid rafts isolation, ganglioside staining, Western blots, and confocal microscopy. BA performed the STED and QD analyses, and graphics. KM-J contributed lipid raft isolation, HPTLC and dot blots. RH-M contributed the work conception, STED and QD experiments, ganglioside staining, confocal microscopy, and wrote manuscript draft. All authors contributed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00027/full#supplementary-material

FIGURE S1 | Specific detection of the ganglioside GT1b in dot blots and sucrose density gradients using a KO-controlled antibody. **(A)** Representative Western blot analysis of sucrose gradient fractions obtained from total membrane homogenates of wild-type brain cortices as described in **Figure 3**. GT1b was detected using a mouse monoclonal anti-GT1b previously characterized (Schnaar et al., 2002). Incubation with the primary antibody (1:1000) was followed by a HRP-conjugated anti-mouse secondary antibody (1:5000). The graphics display the quantification of accumulative distribution of GT1b in bulk membrane fractions (F10 + F11, no lipid rafts: No LR) and in lipid raft fractions (F3 + F4, lipid rafts: LR) (left panel) and in each of the two lipid raft fractions (right panel). **(B)** Dot blot analysis served to demonstrate that GT1b is specifically detected in the wild-type but not detected in *B4Galnt1* KO lipid raft fractions.

REFERENCES

Alvarez, A., Lagos-Cabre, R., Kong, M., Cárdenas, A., Burgos-Bravo, F., Schneider, P., et al. (2016). Integrin-mediated transactivation of P2X7R via hemichannel-dependent ATP release stimulates astrocyte migration. *Biochim. Biophys. Acta* 1863, 2175–2188. doi: 10.1016/j.bbamcr.2016.05.018

Aureli, M., Grassi, S., Sonnino, S., and Prinetti, A. (2016). Isolation and analysis of detergent-resistant membrane fractions. *Methods Mol. Biol.* 1376, 107–131. doi: 10.1007/978-1-4939-3170-5_10

Avalos, A. M., Arthur, W. T., Schneider, P., Quest, A. F., Burridge, K., Leyton, L., et al. (2004). Aggregation of integrins and RhoA activation are required for Thy-1-induced morphological changes in astrocytes. *J. Biol. Chem.* 279, 39139–39145. doi: 10.1074/jbc.M403439200

Avalos, A. M., Valdivia, A. D., Muñoz, N., Herrera-Molina, R., Tapia, J. C., Lavandero, S., et al. (2009). Neuronal Thy-1 induces astrocyte adhesion by engaging syndecan-4 in a cooperative interaction with alphavbeta3 integrin that activates PKCalpha and RhoA. *J. Cell Sci.* 122(Pt 19), 3462–3471. doi: 10.1242/jcs.034827

Barker, T. H., Grenett, H. E., MacEwen, M. W., Tilden, S. G., Fuller, G. M., Settleman, J., et al. (2004). Thy-1 regulates fibroblast focal adhesions, cytoskeletal organization and migration through modulation of p190 RhoGAP and Rho GTPase activity. *Exp. Cell Res.* 295, 488–496. doi: 10.1016/j.yexcr.2004.01.026

Brdicka, T., Cerny, J., and Horejší, V. (1998). T cell receptor signalling results in rapid tyrosine phosphorylation of the linker protein LAT present in detergent-resistant membrane microdomains. *Biochem. Biophys. Res. Commun.* 248, 356–360. doi: 10.1006/bbrc.1998.8857

Bremer, E. G., Schlessinger, J., and Hakomori, S. (1986). Ganglioside-mediated modulation of cell growth. Specific effects of GM3 on tyrosine phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 261, 2434–2440.

Brown, D. A., and Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68, 533–544. doi: 10.1016/0092-8674(92)90189-J

Brugger, B., Graham, C., Leibrecht, I., Mombelli, E., Jen, A., Wieland, F., et al. (2004). The membrane domains occupied by glycosylphosphatidylinositol-anchored prion protein and Thy-1 differ in lipid composition. *J. Biol. Chem.* 279, 7530–7536. doi: 10.1074/jbc.M310207200

Burgos-Bravo, F., Figueroa, N. L., Casanova-Morales, N., Quest, A. F. G., Wilson, C. A. M., Leyton, L., et al. (2018). Single-molecule measurements of the effect of force on Thy-1/alphavbeta3-integrin interaction using nonpurified proteins. *Mol. Biol. Cell* 29, 326–338. doi: 10.1091/mbc.E17-03-0133

Buschiazzo, J., Ialy-Radio, C., Auer, J., Wolf, J. P., Serres, C., Lefèvre, B., et al. (2013). Cholesterol depletion disorganizes oocyte membrane rafts altering mouse fertilization. *PLoS One* 8:e62919. doi: 10.1371/journal.pone.0062919

Chamberlain, L. H. (2004). Detergents as tools for the purification and classification of lipid rafts. *FEBS Lett.* 559, 1–5. doi: 10.1016/S0014-5793(04)00050-X

Chen, X., Jen, A., Warley, A., Lawrence, M. J., Quinn, P. J., and Morris, R. J. (2009). Isolation at physiological temperature of detergent-resistant membranes with properties expected of lipid rafts: the influence of buffer composition. *Biochem. J.* 417, 525–533. doi: 10.1042/BJ20081385

Chen, Y., Thelin, W. R., Yang, B., Milgram, S. L., and Jacobson, K. (2006). Transient anchorage of cross-linked glycosyl-phosphatidylinositol-anchored proteins depends on cholesterol, Src family kinases, caveolin, and phosphoinositides. *J. Cell Biol.* 175, 169–178. doi: 10.1083/jcb.200512116

Chen, Y., Veracini, L., Benistant, C., and Jacobson, K. (2009). The transmembrane protein CBP plays a role in transiently anchoring small clusters of Thy-1, a GPI-anchored protein, to the cytoskeleton. *J. Cell Sci.* 122(Pt 21), 3966–3972. doi: 10.1242/jcs.049346

Chiavegatto, S., Sun, J., Nelson, R. J., and Schnaar, R. L. (2000). A functional role for complex gangliosides: motor deficits in GM2/GD2 synthase knockout mice. *Exp. Neurol.* 166, 227–234. doi: 10.1006/exnr.2000.7504

Choi, J., Leyton, L., and Nham, S. U. (2005). Characterization of alphaX I-domain binding to Thy-1. *Biochem. Biophys. Res. Commun.* 331, 557–561. doi: 10.1016/j.bbrc.2005.04.006

Crespo, P. M., Zurita, A. R., and Daniotti, J. L. (2002). Effect of gangliosides on the distribution of a glycosylphosphatidylinositol-anchored protein in plasma membrane from Chinese hamster ovary-K1 cells. *J. Biol. Chem.* 277, 44731–44739. doi: 10.1074/jbc.M204604200

Danielsen, E. M., and van Deurs, B. (1995). A transferrin-like GPI-linked iron-binding protein in detergent-insoluble noncaveolar microdomains at the apical surface of fetal intestinal epithelial cells. *J. Cell Biol.* 131, 939–950. doi: 10.1083/jcb.131.4.939

Dietrich, C., Volovyk, Z. N., Levi, M., Thompson, N. L., and Jacobson, K. (2001). Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10642–10647. doi: 10.1073/pnas.191168698

Domingues, C. C., Ciana, A., Buttafava, A., Casadei, B. R., Balduini, C., de Paula, E., et al. (2010). Effect of cholesterol depletion and temperature on the isolation of detergent–resistant membranes from human erythrocytes. *J. Membr. Biol.* 234, 195–205. doi: 10.1007/s00232-010-9246-5

Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S., et al. (2009). Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* 457, 1159–1162. doi: 10.1038/nature07596

Fujita, A., Cheng, J., Hirakawa, M., Furukawa, K., Kusunoki, S., and Fujimoto, T. (2007). Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. *Mol. Biol. Cell* 18, 2112–2122. doi: 10.1091/mbc.e07-01-0071

Hanzal-Bayer, M. F., and Hancock, J. F. (2007). Lipid rafts and membrane traffic. *FEBS Lett.* 581, 2098–2104. doi: 10.1016/j.febslet.2007.03.019

Hattersley, K. J., Hein, L. K., and Fuller, M. (2013). Lipid composition of membrane rafts, isolated with and without detergent, from the spleen of a mouse model of Gaucher disease. *Biochem. Biophys. Res. Commun.* 442, 62–67. doi: 10.1016/j.bbrc.2013.11.009

Heffer-Lauc, M., Lauc, G., Nimrichter, L., Fromholt, S. E., and Schnaar, R. L. (2005). Membrane redistribution of gangliosides and glycosylphosphatidylinositol-anchored proteins in brain tissue sections under conditions of lipid raft isolation. *Biochim. Biophys. Acta* 1686, 200–208. doi: 10.1016/j.bbalip.2004.10.002

Heffer-Lauc, M., Viljetic, B., Vajn, K., Schnaar, R. L., and Lauc, G. (2007). Effects of detergents on the redistribution of gangliosides and GPI-anchored proteins in brain tissue sections. *J. Histochem. Cytochem.* 55, 805–812. doi: 10.1369/jhc.7A7195.2007

Hell, S. W. (2007). Far-field optical nanoscopy. *Science* 316, 1153–1158. doi: 10.1126/science.1137395

Henriquez, M., Herrera-Molina, R., Valdivia, A., Alvarez, A., Kong, M., Muñoz, N., et al. (2011). ATP release due to Thy-1-integrin binding induces P2X7-mediated calcium entry required for focal adhesion formation. *J. Cell Sci.* 124(Pt 9), 1581–1588. doi: 10.1242/jcs.073171

Hermosilla, T., Munoz, D., Herrera-Molina, R., Valdivia, A., Muñoz, N., and Nham, S. U., et al. (2008). Direct Thy-1/alphaVbeta3 integrin interaction mediates neuron to astrocyte communication. *Biochim. Biophys. Acta* 1783, 1111–1120. doi: 10.1016/j.bbamcr.2008.01.034

Herrera-Molina, R., Frischknecht, R., Maldonado, H., Seidenbecher, C. I., Gundelfinger, E. D., Hetz, C., et al. (2012). Astrocytic alphaVbeta3 integrin inhibits neurite outgrowth and promotes retraction of neuronal processes by clustering Thy-1. *PLoS One* 7:e34295. doi: 10.1371/journal.pone.0034295

Herrera-Molina, R., Mlinac-Jerkovic, K., Ilic, K., Stober, F., Vemula, S. K., Sandoval, M., et al. (2017). Neuroplastin deletion in glutamatergic neurons impairs selective brain functions and calcium regulation: implication for cognitive deterioration. *Sci. Rep.* 7:7273. doi: 10.1038/s41598-017-07839-9

Herrera-Molina, R., Sarto-Jackson, I., Montenegro-Venegas, C., Heine, M., Smalla, K. H., Seidenbecher, C. I., et al. (2014). Structure of excitatory synapses and GABAA receptor localization at inhibitory synapses are regulated by neuroplastin-65. *J. Biol. Chem.* 289, 8973–8988. doi: 10.1074/jbc.M113.514992

Herrera-Molina, R., Valdivia, A., Kong, M., Alvarez, A., Cardenas, A., Quest, A. F., et al. (2013). Thy-1-interacting molecules and cellular signaling in cis and trans. *Int. Rev. Cell Mol. Biol.* 305, 163–216. doi: 10.1016/B978-0-12-407695-2.00004-4

Honigmann, A., Mueller, V., Ta, H., Schoenle, A., Sezgin, E., Hell, S. W., et al. (2014). Scanning STED-FCS reveals spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells. *Nat. Commun.* 5:5412. doi: 10.1038/ncomms6412

Ilic, K., Mlinac-Jerkovic, K., Jovanov-Milosevic, N., Simic, G., Habek, N., Bogdanovic, N., et al. (2019). Hippocampal expression of cell-adhesion glycoprotein neuroplastin is altered in Alzheimer's disease. *J. Cell Mol. Med.* 23, 1602–1607. doi: 10.1098/rstb.2012.0033

Jia, J.-Y., Lamer, S., Schümann, M., Schmidt, M. R., Krause, E., and Haucke, V. (2006). Quantitative proteomics analysis of detergent–resistant membranes from chemical synapses: evidence for cholesterol as spatial organizer of synaptic vesicle cycling. *Mol. Cell Proteomics* 5, 2060–2071. doi: 10.1074/mcp.M600161-MCP200

Kabouridis, P. S., Janzen, J., Magee, A. L., and Ley, S. C. (2000). Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes. *Eur. J. Immunol.* 30, 954–963. doi: 10.1111/jcmm.13998

Klotzsch, E., and Schutz, G. J. (2013). A critical survey of methods to detect plasma membrane rafts. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368:20120033. doi: 10.1091/mbc.11.5.1645

Kokubo, H., Helms, J. B., Ohno-Iwashita, Y., Shimada, Y., Horikoshi, Y., and Yamaguchi, H. (2003). Ultrastructural localization of flotillin-1 to cholesterol-rich membrane microdomains, rafts, in rat brain tissue. *Brain Res.* 965, 83–90. doi: 10.1016/S0006-8993(02)04140-9

Komura, N., Suzuki, K. G., Ando, H., Konishi, M., Koikeda, M., Imamura, A., et al. (2016). Raft-based interactions of gangliosides with a GPI-anchored receptor. *Nat. Chem. Biol.* 12, 402–410. doi: 10.1038/nchembio.2059

Koumanov, K. S., Tessier, C., Momchilova, A. B., Rainteau, D., Wolf, C., and Quinn, P. J. (2005). Comparative lipid analysis and structure of detergent-resistant membrane raft fractions isolated from human and ruminant erythrocytes. *Arch. Biochem. Biophys.* 434, 150–158. doi: 10.1016/j.abb.2004.10.025

Kusumi, A., Koyama-Honda, I., and Suzuki, K. (2004). Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic* 5, 213–230. doi: 10.1111/j.1600-0854.2004.0178.x

Kusumi, A., Shirai, Y. M., Koyama-Honda, I., Suzuki, K. G. N., and Fujiwara, T. K. (2010). Hierarchical organization of the plasma membrane: investigations by single-molecule tracking vs. fluorescence correlation spectroscopy. *FEBS Lett.* 584, 1814–1823. doi: 10.1016/j.febslet.2010.02.047

Lagos-Cabré, R., Alvarez, A., Kong, M., Burgos-Bravo, F., Cárdenas, A., Rojas-Mancilla, E., et al. (2017). alphaVbeta3 integrin regulates astrocyte reactivity. *J. Neuroinflamm.* 14:194. doi: 10.1186/s12974-017-0968-5

Lagos-Cabré, R., Brenet, M., Díaz, J., Pérez, R. D., Pérez, L. A., Herrera-Molina, R., et al. (2018). Intracellular Ca(2+) increases and connexin 43 hemichannel opening are necessary but not sufficient for Thy-1-induced astrocyte migration. *Int. J. Mol. Sci.* 19:E2179. doi: 10.3390/ijms19082179

Leyton, L., and Hagood, J. S. (2014). Thy-1 modulates neurological cell-cell and cell-matrix interactions through multiple molecular interactions. *Adv. Neurobiol.* 8, 3–20. doi: 10.1007/978-1-4614-8090-7_1

Leyton, L., Schneider, P., Labra, C. V., Rüegg, C., Hetz, C. A., Quest, A. F., et al. (2001). Thy-1 binds to integrin beta(3) on astrocytes and triggers formation of focal contact sites. *Curr. Biol.* 11, 1028–1038. doi: 10.1016/S0960-9822(01)00262-7

Li, H., Turley, S. D., Liu, B., Repa, J. J., and Dietschy, J. M. (2008). GM2/GD2 and GM3 gangliosides have no effect on cellular cholesterol pools or turnover in normal or NPC1 mice. *J. Lipid Res.* 49, 1816–1828. doi: 10.1194/jlr.M800180-JLR200

Lindquist, S., Karitkina, D., Langnaese, K., Posevitz-Fejfar, A., Schraven, B., Xavier, R., et al. (2011). Phosphoprotein associated with glycosphingolipid-enriched microdomains differentially modulates SRC kinase activity in brain maturation. *PLoS One* 6:e23978. doi: 10.1371/journal.pone.0023978

Lingwood, D., and Simons, K. (2007). Detergent resistance as a tool in membrane research. *Nat. Protoc.* 2, 2159–2165. doi: 10.1038/nprot.2007.294

Lingwood, D., and Simons, K. (2010). Lipid rafts as a membrane-organizing principle. *Science* 327, 46–50. doi: 10.1126/science.1174621

Liu, Y., McCarthy, J., and Ladisch, S. (2006). Membrane ganglioside enrichment lowers the threshold for vascular endothelial cell angiogenic signaling. *Cancer Res.* 66, 10408–10414. doi: 10.1158/0008-5472.CAN-06-1572

London, E., and Brown, D. A. (2000). Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim. Biophys. Acta* 1508, 182–195. doi: 10.1016/S0304-4157(00)00007-1

Lopez, P. H., and Schnaar, R. L. (2009). Gangliosides in cell recognition and membrane protein regulation. *Curr. Opin. Struct. Biol.* 19, 549–557. doi: 10.1016/j.sbi.2009.06.001

Mahanthappa, N. K., and Patterson, P. H. (1992). Thy-1 multimerization is correlated with neurite outgrowth. *Dev. Biol.* 150, 60–71. doi: 10.1016/0012-1606(92)90007-4

Maldonado, H., Calderon, C., Burgos-Bravo, F., Kobler, O., Zuschratter, W., Ramirez, O., et al. (2017). Astrocyte-to-neuron communication through integrin-engaged Thy-1/CBP/Csk/Src complex triggers neurite retraction via the RhoA/ROCK pathway. *Biochim. Biophys. Acta Mol. Cell. Res.* 1864, 243–254. doi: 10.1016/j.bbamcr.2016.11.006

Marushchak, D., Gretskaya, N., Mikhalyov, I., and Johansson, L. B. (2007). Self-aggregation–an intrinsic property of G(M1) in lipid bilayers. *Mol. Membr. Biol.* 24, 102–112. doi: 10.1080/09687860600995235

Mlinac, K., Jovanov, N., Milosevic, M., Heffer, Smalla, K. H., Schnaar, R. L., et al. (2012). Neuroplastin expression in the hippocampus of mice lacking complex gangliosides. *J. Mol. Neurosci.* 48, 161–166. doi: 10.1007/s12031-012-9801-x

Morris, R. J., Jen, A., and Warley, A. (2011). Isolation of nano-meso scale detergent resistant membrane that has properties expected of lipid 'rafts'. *J. Neurochem.* 116, 671–677. doi: 10.1111/j.1471-4159.2010.07076.x

Mukherjee, P., Faber, A. C., Shelton, L. M., Baek, R. C., Chiles, T. C., and Seyfried, T. N. (2008). Thematic review series: sphingolipids. *Ganglioside GM3* suppresses the proangiogenic effects of vascular endothelial growth factor and ganglioside GD1a. *J. Lipid Res.* 49, 929–938. doi: 10.1194/jlr.R800006-JLR200

Mutoh, T., Tokuda, A., Miyadai, T., Hamaguchi, M., and Fujiki, N. (1995). Ganglioside GM1 binds to the Trk protein and regulates receptor function. *Proc. Natl. Acad. Sci. U.S.A.* 92, 5087–5091. doi: 10.1073/pnas.92.11.5087

Ohmi, Y., Tajima, O., Ohkawa, Y., Mori, A., Sugiura, Y., Furukawa, K., et al. (2009). Gangliosides play pivotal roles in the regulation of complement systems and in the maintenance of integrity in nerve tissues. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22405–22410. doi: 10.1073/pnas.0912336106

Owen, D. M., Magenau, A., Williamson, D., and Gaus, K. (2012). The lipid raft hypothesis revisited–new insights on raft composition and function from super-resolution fluorescence microscopy. *Bioessays* 34, 739–747. doi: 10.1002/bies.201200044

Pereira, D. B., and Chao, M. V. (2007). The tyrosine kinase Fyn determines the localization of TrkB receptors in lipid rafts. *J. Neurosci.* 27, 4859–4869. doi: 10.1523/JNEUROSCI.4587-06.2007

Persaud-Sawin, D. A., Lightcap, S., and Harry, G. J. (2009). Isolation of rafts from mouse brain tissue by a detergent-free method. *J. Lipid Res.* 50, 759–767. doi: 10.1194/jlr.D800037-JLR200

Pike, L. J. (2009). The challenge of lipid rafts. *J. Lipid Res.* 50(Suppl), S323–S328. doi: 10.1194/jlr.R800040-JLR200

Pike, L. J., Han, X., Chung, K. N., and Gross, R. W. (2002). Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry* 41, 2075–2088. doi: 10.1021/bi0156557

Polyakova, O., Dear, D., Stern, I., Martin, S., Hirst, E., Bawumia, S., et al. (2009). Proteolysis of prion protein by cathepsin S generates a soluble beta-structured intermediate oligomeric form, with potential implications for neurotoxic mechanisms. *Eur. Biophys. J.* 38, 209–218. doi: 10.1007/s00249-008-0371-3

Prinetti, A., Loberto, N., Chigorno, V., and Sonnino, S. (2009). Glycosphingolipid behaviour in complex membranes. *Biochim. Biophys. Acta* 1788, 184–193. doi: 10.1016/j.bbamem.2008.09.001

Pryor, S., McCaffrey, G., Young, L. R., and Grimes, M. L. (2012). NGF causes TrkA to specifically attract microtubules to lipid rafts. *PLoS One* 7:e35163. doi: 10.1371/journal.pone.0035163

Quest, A. F. G., Leyton, L., and Párraga, M. (2004). Caveolins, caveolae, and lipid rafts in cellular transport, signaling, and disease. *Biochem. Cell Biol.* 82, 129–144. doi: 10.1139/o03-071

Richter, K. N., Revelo, N. H., Seitz, K. J., Helm, M. S., Sarkar, et al. (2018). Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy. *EMBO J.* 37, 139–159. doi: 10.15252/embj.201695709

Samuel, B. U., Mohandas, N., Harrison, T., McManus, H., Rosse, W., Reid, M., et al. (2001). The role of cholesterol and glycosylphosphatidylinositol-anchored

proteins of erythrocyte rafts in regulating raft protein content and malarial infection. *J. Biol. Chem.* 276, 29319–29329. doi: 10.1074/jbc.M101268200

Santos, A. L., and Preta, G. (2018). Lipids in the cell: organisation regulates function. *Cell Mol. Life Sci.* 75, 1909–1927. doi: 10.1007/s00018-018-2765-4

Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993). Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* 122, 789–807. doi: 10.1083/jcb.122.4.789

Schnaar, R. L., Fromholt, S. E., Gong, Y., Vyas, A. A., Laroy, W., Wayman, D. M., et al. (2002). Immunoglobulin G-class mouse monoclonal antibodies to major brain gangliosides. *Anal. Biochem.* 302, 276–284. doi: 10.1006/abio.2001.5540

Schnaar, R. L., Gerardy-Schahn, R., Hildebrandt, H. (2014). Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiol. Rev.* 94, 461–518. doi: 10.1152/physrev.00033.2013

Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., and Simons, K. (2003). Resistance of cell membranes to different detergents. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5795–5800. doi: 10.1073/pnas.0631579100

Sezgin, E. (2017). Super-resolution optical microscopy for studying membrane structure and dynamics. *J. Phys. Condens. Matter.* 29:273001. doi: 10.1088/1361-648X/aa7185

Sharma, P., Varma, R., Sarasij, R. C., Ira, Gousset, K., Krishnamoorthy, G., Rao, M., et al. (2004). Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* 116, 577–589. doi: 10.1016/S0092-8674(04)00167-9

Sheikh, K. A., Sun, J., Liu, Y., Kawai, H., Crawford, T. O., Proia, R. L., et al. (1999). Mice lacking complex gangliosides develop Wallerian degeneration and myelination defects. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7532–7537. doi: 10.1073/pnas.96.13.7532

Silvius, J. R. (2003). Role of cholesterol in lipid raft formation: lessons from lipid model systems. *Biochim. Biophys. Acta* 1610, 174–183. doi: 10.1016/S0005-2736(03)00016-6

Simons, K., and Ikonen, E., (1997). Functional rafts in cell membranes. *Nature* 387, 569–572. doi: 10.1038/42408

Simons, M., Friedrichson, T., Schulz, J. B., Pitto, M., Masserini, M., and Kurzchalia, T. V. (1999). Exogenous administration of gangliosides displaces GPI-anchored proteins from lipid microdomains in living cells. *Mol. Biol. Cell* 10, 3187–3196. doi: 10.1091/mbc.10.10.3187

Skibbens, J. E., Roth, M. G., and Matlin, K. S. (1989). Differential extractability of influenza virus hemagglutinin during intracellular transport in polarized epithelial cells and nonpolar fibroblasts. *J. Cell Biol.* 108, 821–832. doi: 10.1083/jcb.108.3.821

Solheim, S. A., Petsalaki, E., Stokka, A. J., Russell, R. B., Taskén, K., Berge, T., et al. (2008). Interactions between the Fyn SH3-domain and adaptor protein Cbp/PAG derived ligands, effects on kinase activity and affinity. *FEBS J.* 275, 4863–4874. doi: 10.1111/j.1742-4658.2008.06626.x

Sones, W. R., Davis, A. J., Leblanc, N., and Greenwood, I. A. (2010). Cholesterol depletion alters amplitude and pharmacology of vascular calcium-activated chloride channels. *Cardiovasc. Res.* 87, 476–484. doi: 10.1093/cvr/cvq057

Sonnino, S., and Prinetti, A. (2013). Membrane domains and the "lipid raft" concept. *Curr. Med. Chem.* 20, 4–21.

Sonnino, S, Mauri, L., Chigorno, V., and Prinetti, A. (2007). Gangliosides as components of lipid membrane domains. *Glycobiology* 17, 1R–13R. doi: 10.1093/glycob/cwl052

Suzuki, K. G., Fujiwara, T. K., Edidin, M., and Kusumi, A. (2007a). Dynamic recruitment of phospholipase C gamma at transiently immobilized GPI-anchored receptor clusters induces IP3-Ca2+ signaling: single-molecule tracking study 2. *J. Cell Biol.* 177, 731–742. doi: 10.1083/jcb.200609175

Suzuki, K. G., Fujiwara, T. K., Sanematsu, F., Iino, R., Edidin, M., and Kusumi, A. (2007b). GPI-anchored receptor clusters transiently recruit Lyn and G alpha for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J. Cell Biol.* 177, 717–730. doi: 10.1083/jcb.200609174

Suzuki, K. G. N., Ando, H., Komura, N., Fujiwara, T. K., Kiso, M., and Kusumi, A. (2017). Development of new ganglioside probes and unraveling of raft domain structure by single-molecule imaging. *Biochim. Biophys. Acta* 1861, 2494–2506. doi: 10.1016/j.bbagen.2017.07.012

Suzuki, S., Numakawa, T., Shimazu, K., Koshimizu, H., Hara, T., Hatanaka, H., et al. (2004). BDNF-induced recruitment of TrkB receptor into neuronal lipid rafts: roles in synaptic modulation. *J. Cell Biol.* 167, 1205–1215. doi: 10.1083/jcb.200404106

Svennerholm, L. (1963). Chromatographic separation of human brain gangliosides. *J. Neurochem.* 10, 613–623. doi: 10.1111/j.1471-4159.1963.tb08933.x

Svennerholm, L. (1980). Ganglioside designation. *Adv. Exp. Med. Biol.* 125:11. doi: 10.1007/978-1-4684-7844-0_2

Takamiya, K., Yamamoto, A., Furukawa, K., Yamashiro, S., Shin, M., Okada, M., et al. (1996). Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10662–10667. doi: 10.1073/pnas.93.20.10662

Takamiya, K., Yamamoto, A., Furukawa, K., Zhao, J., Fukumoto, S., Yamashiro, S., et al. (1998). Complex gangliosides are essential in spermatogenesis of mice: possible roles in the transport of testosterone. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12147–12152. doi: 10.1073/pnas.95.21.12147

Tanaka, K. A., Suzuki, K. G., Shirai, Y. M., Shibutani, S. T., Miyahara, M. S., Tsuboi, H., et al. (2010). Membrane molecules mobile even after chemical fixation. *Nat. Methods* 7, 865–866. doi: 10.1038/nmeth.f.314

Vajn, K., Viljetić, B., Degmečić, I. V., Schnaar, R. L., and Heffer, M. (2013). Differential distribution of major brain gangliosides in the adult mouse central nervous system. *PLoS One* 8:e75720. doi: 10.1371/journal.pone.0075720

van Zanten, T. S., Gomez, J., Manzo, C., Cambi, A., Buceta, J., Reigada, R., et al. (2010). Direct mapping of nanoscale compositional connectivity on intact cell membranes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15437–15442. doi: 10.1073/pnas.1003876107

Williamson, R., Thompson, A. J., Abu, M., Hye, A., Usardi, A., Lynham, S., et al. (2010). Isolation of detergent resistant microdomains from cultured neurons: detergent dependent alterations in protein composition. *BMC Neurosci.* 11:120. doi: 10.1186/1471-2202-11-120

Wong, L., Lieser, S. A., Miyashita, O., Miller, M., Tasken, K., Onuchic, J. N., et al. (2005). Coupled motions in the SH2 and kinase domains of Csk control Src phosphorylation. *J. Mol. Biol.* 351, 131–143. doi: 10.1016/j.jmb.2005.05.042

Yang, S. H., Chen, Y. J., Tung, P. Y., Lai, W. L., Chen, Y., Jeng, C. J., et al. (2008). Anti-Thy-1 antibody-induced neurite outgrowth in cultured dorsal root ganglionic neurons is mediated by the c-Src-MEK signaling pathway. *J. Cell. Biochem.* 103, 67–77. doi: 10.1002/jcb.21387

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92, 83–92. doi: 10.1016/S0092-8674(00)80901-0

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Thy-1 Deficiency Augments Bone Loss in Obesity by Affecting Bone Formation and Resorption

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Healthy bone remodeling results from a balanced bone formation and bone resorption realized by bone-forming osteoblasts and bone-resorbing osteoclasts, respectively. Recently, Thy-1 (CD90) was identified as positive regulator of osteoblast differentiation and activation, thus, promoting bone formation while concurrently inhibiting adipogenesis and obesity in mice. Additionally, Thy-1 did not affect bone resorption. An obesity-related co-morbidity that is increasing in prevalence is a disturbed bone formation resulting in an increased fracture risk. The underlying mechanisms of obesity-induced bone alterations are not yet fully elucidated and therefore therapy options for efficient bone-anabolic treatments are limited. Therefore, we investigated the impact of Thy-1 on bone metabolism under obese conditions. Indeed, high fat diet (HFD) induced obese mice lacking Thy-1 (Thy-$1^{-/-}$) showed increased body fat mass compared to wildtype (WT) mice while bone mass (−38%) and formation (−57%) were decreased as shown by micro-computed tomography (μCT) measurement, histological analysis, and fourier-transform infrared spectroscopy (FTIR). Interestingly, under obese conditions, lack of Thy-1 affected both osteoblast and osteoclast function. Number (−30%) and activity of osteoblasts were decreased in obese Thy-$1^{-/-}$ mice while osteoclast number (+39%) and activity were increased. Facilitated bone marrow fat accumulation (+56%) in obese Thy-$1^{-/-}$ mice compared to obese WT mice was associated with upregulated tumor necrosis factor α (*Tnf*α, +46%) and colony stimulating factor 1 receptor *(Csf1r)* expression, strong promoters of osteoclast differentiation. Moreover, lack of Thy-1 was accompanied by a reduction of osteoprotegerin (*Tnfrsf11b*) expression (−36%), an inhibitor of osteoclast differentiation. Altered *Tnf*α, *Csf1r,* and *Tnfrsf11b* expression might be responsible for elevated osteoclast activity in obese Thy-1-deficient mice. In summary, our findings show that lack of Thy-1 promotes obesity under HFD conditions while concurrently decreasing bone mass and formation. Mechanistic studies revealed that under obese conditions lack of Thy-1 impairs both bone formation and bone resorption.

Keywords: obesity, Thy-1, bone mass, osteoblast, osteoclast, adipocytes, differentiation, TNFα

INTRODUCTION

Healthy bone remodeling is a result of balanced bone formation realized bone-forming osteoblasts and bone-resorption mediated by bone resorbing osteoclasts (Crockett et al., 2011). Recently, Thy-1 (CD90) was identified as a critical molecule for the differentiation of osteoblasts and, thus, promoting osteogenesis and bone formation while inhibiting adipogenesis and obesity. Thy-1 is a glycosylphosphatidyl-anchored protein located on the cell surface of mesenchymal stem cells (MSCs), fibroblasts, activated microvascular endothelial cells, neurons, a subpopulation of hematopoietic stem cells (HSCs) and mouse T-cells (Vitetta et al., 1973; Craig, 1993; Wetzel et al., 2004; Schmidt et al., 2015; Picke et al., 2018a). Recently, it was discovered that Thy-1 controls fate decision of MSCs regarding differentiation into mature bone-forming osteoblasts or fat-storing adipocytes *in vitro* and *in vivo* (Hosoya et al., 2012; Chung et al., 2013; Woeller et al., 2015; Paine et al., 2018; Picke et al., 2018a). Mice lacking Thy-1 display a reduced osteogenic and increased adipogenic differentiation capacity, resulting in decreased bone mass and quality and concurrently, elevated body and bone marrow fat mass (Woeller et al., 2015; Paine et al., 2018; Picke et al., 2018a). Reduced osteogenesis in Thy-1 deficiency was linked to increased serum concentrations of the Wnt signaling inhibitors Dickkopf-1 (Dkk-1) and sclerostin, diminished Wnt ligand expression and attenuated Wnt signaling (Picke et al., 2018a). However, Thy-1 did not affect bone resorption in lean mice. The translational potential of these findings was underlined by the detection of strongly reduced levels of soluble Thy-1 in serum of patients with diminished bone formation such as in osteoporotic and obese patients (Picke et al., 2018a).

Worldwide, obesity is a major health problem negatively affecting bone metabolism. Obese patients often show increased bone mass and paradoxically suffer from an highly elevated fracture risk (Kling et al., 2014; Greco et al., 2015). Interestingly, in obesity bone remodeling is elevated in early phases due to increased mechanical loading by high body weight, which results in elevated bone mass (Greco et al., 2015). Later on, the massive amount of adipose tissue, especially bone marrow fat and visceral fat depots, leads to an increased production of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) resulting in chronic inflammatory response (Hotamisligil, 2006; Sharma et al., 2014; Palermo et al., 2016). This abnormal cytokine production results in altered bone mass and highly increased fracture risk in obese patients (Hsu et al., 2006; Nielson et al., 2012; Greco et al., 2015; Palermo et al., 2016).

Tumor necrosis factor α has the potential to increase the osteogenic differentiation capacity of MSCs or to reduce the osteogenic differentiation of pre-osteoblasts, which have already started their differentiation process (Gilbert et al., 2000; Osta et al., 2014). In addition, TNFα also promotes the differentiation of HSCs into osteoclasts by promoting actin ring formation and inducing an elevated secretion of receptor activator of NF-κB ligand (RANKL) by osteoblasts (Fuller et al., 2002; Osta et al., 2014). RANKL binds to its receptor RANK, located at the surface of osteoclast precursor cells, resulting in an increase in osteoclastogenesis. TNFα and RANKL have also been shown to operate synergistically on osteoclastogenesis by increasing RANK expression via TNF type 1 receptor (TNFR1) signaling (Zhang et al., 2001). In addition, CSF1, produced by osteoblasts, supports the positive effect of TNFα on osteoclastogenesis. Consequently, inhibition of CSF-1 in mice resulted in reduced osteoclastogenesis and osteolysis (Kitaura et al., 2005). Osteoblasts also produce osteoprotegerin (OPG), a decoy receptor of RANKL, and can therefore inhibit osteoclast differentiation (Boyce and Xing, 2008). In mice, high fat diet (HFD) reduces bone mass due to increased bone marrow adipogenesis and osteoclastogenesis mediated by higher levels of TNFα, RANKL, and PPARγ (Shu et al., 2015). The underlying mechanisms of HFD induced bone alterations are not yet fully elucidated and therefore, therapy options for efficient bone-anabolic treatments are limited (Tu et al., 2018).

In the present study, we analyzed the impact of Thy-1 on disturbed bone metabolism in obesity. Thy-1-deficient (Thy-$1^{-/-}$) and wildtype (WT) mice were fed with a HFD to induce obesity. We detected a reduced number and activity of osteoblasts resulting in a decreased bone formation in obese Thy-$1^{-/-}$ mice. In parallel, in obese Thy-$1^{-/-}$ mice, osteoclast number and activity were increased. An elevated bone marrow adiposity associated with an increased pro-inflammatory environment including increased *TNFa* and *Csf1r* expression and an attenuated gene expression of OPG (*Tnfsf11B*), the decoy receptor for RANKL (*Tnfsf11*) in obese Thy-$1^{-/-}$ mice contributed to strengthened bone resorption. Thus, under obese conditions, Thy-1 affects both the osteo-anabolic and -catabolic metabolism.

MATERIALS AND METHODS

Mice

Thy-1 deficient (KO) mice on C57BL/6J background [kindly provided as a gift from Dr. R. Morris King's College London, (61)] and C57BL/6J wildtype (WT) mice were kept under a 12-h light-dark cycle and given food and water *ad libitum*. All animal experiments were performed in accordance with institutional and state guidelines and approved by the Committee on Animal Welfare of Saxony (TVV 03/16, T26/16). Four to five weeks old male C57BL/6J mice were fed a HFD (EF R/M D12331 diet modified by Surwit, ssniff, Soest, Germany). The second cohort (**Supplementary Figure 1**; Picke et al., 2018a) were fed a standard chow until the age of 12 weeks.

RNA Isolation, RT, and Quantitative Real Time PCR (qRT-PCR)

Total RNA from bone samples (ulnae) was isolated using a Trifast (PEQLAB, United States) method following the manufacturer's instructions. For first strand cDNA synthesis with M-MLV reverse transcriptase (Promega, Madison, WI, United States), 1 μg total RNA was used according to the manufacturer's protocol. Using *GoTaq® qPCR Master* (Promega) the real-time qPCR was performed according to the manufacturer's instructions on Rotor-Gene Q (QIAGEN). Used primers are listed in **Supplementary Table 1**. Quantitative gene expression

was calculated from the standard curve of cloned cDNA and was normalized to the unregulated reference genes *Rs36* (*ex vivo* cell culture) *or Gapdh* (bones).

Serum Analysis

Blood was drawn by heart puncture, centrifuged and frozen at −80°C. Serum levels of type 1 procollagen amino-terminal propeptide (P1NP), C-terminal telopeptide (CTX and P1NP: Immundiagnostik Systems, Germany), sclerostin (ALPCO, serum dilution 1:5), and Dkk-1 (R&D) were detected using immunoassay kits according to the manufacturer's protocols.

Assessment of Bone Mass, Microarchitecture, and Bone Marrow Fat Volume

Extracted bones were fixed for 48 h in 4% paraformaldehyde (PFA, Carl Roth, Karlsruhe, Germany) and were afterward dehydrated in 80% ethanol. By using the μCT vivaCT 40 (isotropic voxel size of 10.5 μm; 70 kVp, 114 μA, 200 ms integration time, Scanco Medical, Switzerland), femora and third lumbar vertebral bodies were analyzed as previously described (Picke et al., 2018a). The analysis of trabecular and cortical bone volume per total volume (BV/TV), bone mineral density (BMD), thickness (Tb.Th and Ct.Th for the trabecular and cortical thickness, respectively), trabecular number (Tb.N), trabecular separation (Tb.Sp) and cortical porosity (Ct.Po) was performed using established analysis protocols and the μCT parameters were reported according to international guidelines (63). To analyze the total area (Tt.Ar), marrow area (Ma.Ar) and cortical bone area (Ct.Ar) the periosteal and endosteal surfaces at the femoral mid-shaft were identified and afterward computed as the area within the periosteal boarder, the area within the endosteal border, and the area between the periosteal and endosteal borders, respectively (Picke et al., 2018a). Animations of the trabecular microstructure were generated from the Digital Imaging and Communications in Medicine (DICOM) image files in Amira (v6.0.0, Thermo Fisher Scientific, Hillsboro, OR, United States) and saved in gif format using ImageJ (v 1.46 r, NIH, United States; see **Supplementary Animation 1** of the trabecular bone compartment of femur of either WT or KO mice).

For assessment of the bone marrow fat content, fixed femora were decalcified (OSTEOSOFT®) for seven d and afterward scanned via μCT to ensure complete decalcification. They were then washed with PBS for 5 min, stained for 2 h with 2% osmium tetroxide (Electron Microscopy Science, United Kingdom) diluted in 0.1 M sodium cacodylate buffer (pH 7.4) (Picke et al., 2016), and were transferred into PBS. The complete femur was analyzed with μCT (70 kVp, 114 μA, 300 ms integration time, 10.5 μm isotropic voxel size) to evaluate the fat volume using the established protocols from Scanco Medical as previously reported (Picke et al., 2018a).

Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

Fourier-transform infrared spectroscopy spectra was measured at a Spotlight 400 (PerkinElmer, Waltham, MA, United States) attached to a Frontier FTIR spectrometer (PerkinElmer, Waltham, MA, United States) in ATR-mode. Spectra were acquired within a wavelength range from 4000 to 570 cm^{-1} with a resolution of 4 cm^{-1}. For each pixel (6.25 μm × 6.25 μm), 16 measurements were taken. SpectrumIMAGE software R.1.8.0.0410 (PerkinElmer, Waltham, MA, United States) was used for automatic background subtraction for each pixel spectrum and for automatic ATR correction. Each region of interest was 300 × 300 μm^2 in size and included the whole cortical thickness. The spectra were post processed using a customized MATLAB (MATLAB 2014b, The MathWorks, Inc., MA, United States) routine with PMMA subtraction and baseline correction. Crystallinity was calculated by sub-peak fitting of the entire phosphate peak (1154–900 cm^{-1}) and calculation of the ratio of the 1030 and 1020 cm^{-1} sub-peaks (27). The sub-peak of 1020 cm^{-1} is linked to non-stoichiometric apatite, whereas the sub-peak of 1030 cm^{-1} is linked to stoichiometric apatite (64). An increase of the crystallinity ratio reflects either an increase of stoichiometric apatite or a decrease of non-stoichiometric apatite (i.e., the crystallinity ratio reflects crystal size and perfection).

Reference-Point Analysis, Three-Point Bending Test, and Femur Properties

The femora were shock frozen in liquid nitrogen after sacrifice and thawed from −80°C 5 min before reference-point indentation™ (BioDent2, Active Life Scientific, United States). To avoid sample drying the bones were stabilized using an *ex vivo* small bone stage, which was filled with PBS. The reference probe was located at the anterior side of the femur shaft and indentation measurements were performed (2 N, five cycles) in triplicates for each bone sample by lifting up the measurement head unit and keeping the movement of the sample to a minimum. The total distance increase (TDI) was calculated. Promptly afterward, the femora were mechanically tested in a three-point bending (zwicki-Line 2.5 kN, Zwick, Germany). Load was applied at the anterior site of the femoral shaft until failure and the maximum load (F_{max}, N) was recorded as previously reported (Picke et al., 2016, 2018a). The length (proximal to distal: greater trochanter to condyles) and width (shaft) of femora were measured using a caliper.

Bone Histology and Histomorphometry

WT and Thy-1-deficient mice received i.p. injections of calcein (20 mg/kg) 5 and 2 days prior to sacrifice as previously reported (Picke et al., 2018a). The right and left proximal tibia were fixed in 4% PFA and dehydrated with 80% ethanol. For examination of bone formation rate by analysis of calcein labels (fluorescence), left tibiae were embedded in methyl methacrylate (Technovit 9100, Heraeus Kulzer, GER) and cut into 7-μm sections. BV/TV, bone formation rate per bone surface (BFR/BS), mineralized surface per BS (MS/BS), and mineral apposition rate (MAR) were determined in the trabecular part of the bone using the OsteoMeasure® Software (OsteoMetrics, Atlanta, GA, United States) following the international standards (Parfitt et al., 1987). Mineralized bone areas (BV/TV) and osteoid surface per bone perimeter (Osteoid.S/B.Pm) were visualized by *von*

Kossa staining and *van Gieson* counter staining. Therefore, bone sections were rehydrated using decreasing alcohol concentrations and were then sequentially exposed to silver nitrate (Roth), sodium carbonate (Merck), and sodium thiosulfate (Roth). Afterward, the slides were stained with toluidine blue (Waldeck GmbH, Germany) and dehydrated. The right proximal tibiae were then decalcified using OSTEOSOFT® and embedded in paraffin (Leica Biosystems, United States) for the analysis of osteoclast numbers per bone surface (Oc.N/BS) and osteoclast surface per bone perimeter (Oc.S/B.Pm). Bone slices of 2 μm were stained at 37°C with tartrate resistant acid phosphatase (TRAP) staining solution (Naphthol-AS-BI-Phosphate, Fast Red Violet LB Salt, Triton X-100, dimethylformamide) and hemalum (Picke et al., 2018a).

Ex vivo Experiments

Mesenchymal stem cells were isolated from femora and tibiae of Thy-1-deficient and WT mice by enzymatic digestion using 26 U/ml of Liberase DL (Roche) for 2 h at 37°C and 5% CO_2. Cell suspension was filtered through a cell strainer (70 μm) and cultured in a-MEM medium (Lonza) supplemented with 10% fetal calf serum (FCS) (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Biochrom AG) at 37°C, 5% CO_2. CD11b-positive cells were removed by magnetic cell separation using anti-CD11b magnetic beads according to the manufacturer's protocol (Miltenyi). Purity was checked as described previously (Picke et al., 2018a). MSCs were stimulated with 10 ng/ml TNF (Miltenyi) for 24 h.

Statistical Analysis

The results are presented as mean ± standard deviation (SD). Distribution of data was assessed by Shapiro-Wilk test. Depending on the normality of the data, analysis was performed using the Mann-Whitney rank-sum test or the *t*-test. For assessment of the effect of Thy-1 deficiency and TNFα treatment on MSCs *ex vivo*, we performed two-way ANOVA with Tukey *post hoc* test. $P < 0.05$ was considered significant.

RESULTS

Obese Thy-1$^{-/-}$ Mice Display Decreased Trabecular Bone Mass While Cortical Bone Mass and Biomechanical Properties Are Unaltered

Since diet-induced obesity alters bone remodeling leading to decreased femoral trabecular bone mass in mice (Cao et al., 2009, 2010; Halade et al., 2011; Patsch et al., 2011; Picke et al., 2018b), we investigated the impact of Thy-1 on the disturbed bone remodeling in obesity. A significantly higher weight gain was observed beginning 12 weeks after starting a HFD in Thy-1$^{-/-}$ compared to WT mice. As control, one group of WT mice was fed a standard chow diet (CHOW, **Figure 1A**). The impact of Thy-1 deficiency on characteristics of the femoral and tibial long bones as well as of lumbar vertebral bodies after 18 weeks of HFD was analyzed. Similar to mice fed a HFD for 8 weeks (Paine et al., 2018), lack of Thy-1 reduced femoral trabecular bone mass (**Figure 1B** and **Supplementary Animation 1** and **Supplementary Table 2A**) due to a decreased trabecular number and increased trabecular separation (**Supplementary Table 2A**). In addition, we could show that in contrast to lean mice, Thy-1 deficiency also reduced bone mass of the lumbar vertebral bodies under obese conditions (**Supplementary Figure 1** and **Supplementary Table 2D**).

Because HFD-induced obesity often results in an elevated cortical bone mass in humans while fracture risk is highly increased (Cobayashi et al., 2005; Hsu et al., 2006; Zhao et al., 2007; Hardcastle et al., 2015), we analyzed the bone mass and biomechanical properties of the cortical bone compartment. The cortical bone mass and cortical thickness were not altered in obese Thy-1$^{-/-}$ mice (**Figure 1B** and **Supplementary Table 2B**). Cortical porosity was not affected by Thy-1 deficiency after HFD (**Figure 1C**). Using reference-point indentation, we detected no differences between WT and Thy-1$^{-/-}$ mice regarding the total indentation distance (TID, **Figure 1D**), a marker for hardness of cortical bone (Thurner, 2009). In line with that, the moment of inertia (MOI, **Figure 1E**), which is a geometrical parameter that is indicative of resistance to load, and the total tissue area, cortical area, and marrow area (Tt.Ar, Ct.Ar, Ma.Ar, **Supplementary Table 2B**) as well as the femur length and width (**Supplementary Table 2C**) were likewise unaffected by Thy-1 deficiency after HFD. By performing a three-point bending test, we could validate the results of the geometric measurement. The same force was needed to fracture the femora from WT and Thy-1$^{-/-}$ mice (**Figure 1F**). Moreover, in Thy-1$^{-/-}$ mice, the collagen amount within the bone matrix was decreased, indicated by an elevated mineral-to-matrix ratio (MMR, +8%, **Figure 1G**) analyzed by FTIR (Boskey and Pleshko Camacho, 2007). Taking into account the decreased tissue mineral density measured by μCT (**Supplementary Table 2**), the mineral age was younger shown by a reduced carbonate-to-phosphate ratio (CPR, −5%, **Figure 1H**).

In summary, in obesity, lack of Thy-1 resulted in reduced bone mass while bone stiffness was not affected.

Lack of Thy-1 Reduces Osteoblast Differentiation as Well as Bone Formation and Increases Osteoclast Differentiation and Bone Resorption in Obese Mice

Bone remodeling is a result of the balanced activity of bone-forming osteoblasts and bone-resorbing osteoclasts resulting in a similar bone formation and resorption rate (Crockett et al., 2011). Therefore, both the differentiation and activity of osteoblasts and osteoclasts were analyzed in obese Thy-1$^{-/-}$ and obese WT mice.

Concerning bone formation, we observed a diminished number, differentiation and activity of osteoblasts (**Figures 2A–J**). The number of osteoblasts (Ob.N/B.Pm, −30%, **Figure 2A**) was decreased in obese Thy-1$^{-/-}$ mice, while osteoblast surface per bone surface (Ob.S/BS, **Figure 2B**) showed trends toward a reduction compared to the WT mice. In

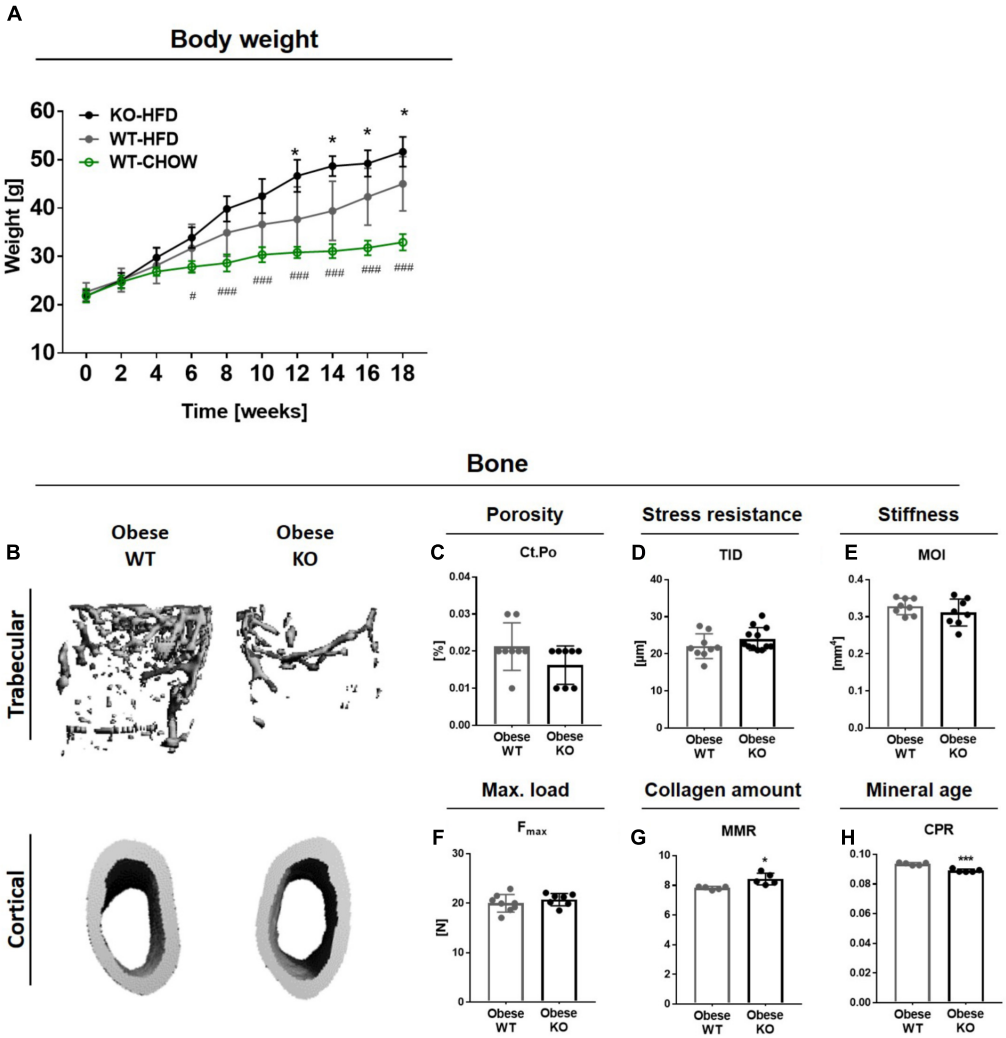

FIGURE 1 | Obese Thy-$1^{-/-}$ mice display decreased trabecular bone mass while cortical bone mass and biomechanical properties are unaltered. Wildtype (WT) and Thy-$1^{-/-}$ (KO) mice were fed with a high fat diet for 18 weeks (HFD). As control group, WT mice were fed a standard chow (CHOW) for the same time period. **(A)** The body weight of standard and HFD fed WT and KO mice over 18 weeks. Hashtags denote significance level of #$P < 0.05$, ###$P < 0.001$ between WT-HFD and WT-CHOW and asterisks denote significance level of *$P < 0.05$ between WT-HFD and KO-HFD (Student's *t*-test). **(B)** Representative 3D-images of the trabecular (upper row) and cortical bone compartment (lower row) of the femur. Bone values are presented in **Supplementary Table 2**. **(C)** Cortical porosity (Ct.Po), **(D)** total indentation distance (TID, reference-point indentation), **(E)** moment of inertia (MOI, µCT calculation), and **(F)** maximum force (F_{max}, three-point bending test) to fracture cortical bone were examined. **(G)** Mineral-to-matrix ratio (MMR) and **(H)** carbonate-to-phosphate ratio were analyzed using fourier-transform infrared spectroscopy (FTIR). Each point represents one mouse and median ± SD is presented. Asterisks denote significance level of *$P < 0.05$, ***$P < 0.001$ (Student's *t*-test).

accordance, the gene expression of the osteogenic markers *Runx2* (−27%, **Figure 2C**) and *Tnalp* (−60%, **Figure 2D**) were decreased in bones of obese Thy-$1^{-/-}$ mice. Reduced serum concentration of the bone formation marker P1NP (−27%, **Figure 2E**) indicated an attenuated osteoblast activity. Decreased osteoid surface (OS/B.Pm, −43%, **Figures 2F,G**), which indicates impaired mineralization, and decreased bone formation rate (BFR/BS, −57%, **Figures 2H,J**) due to a lower mineral apposition rate (MAR, −59%, **Figures 2I,J**), substantiated these findings. These data were validated in the lumbar vertebral body shown by a reduced bone formation rate and mineral surface per bone surface (BFR/BS, −51%, MS/BS, −46%, **Supplementary Table 2F**).

In parallel to the analysis of osteoblast differentiation and activity in obese Thy-$1^{-/-}$ mice, we examined the impact of Thy-1 on bone resorption. Indeed, osteoclast number (Oc.N/B.Pm, +39%, **Figures 2K,M**) and surface (Oc.S/BS, +36%, **Figures 2L,M**) were increased in the tibia and lumbar vertebral body (**Supplementary Table 2G**) in obese Thy-$1^{-/-}$ mice. Additionally, the concentration of CTX, a serum bone resorption marker, was elevated (+30%, **Figure 2N**). Correspondingly, we observed an elevated gene expression of *Trap*, a marker

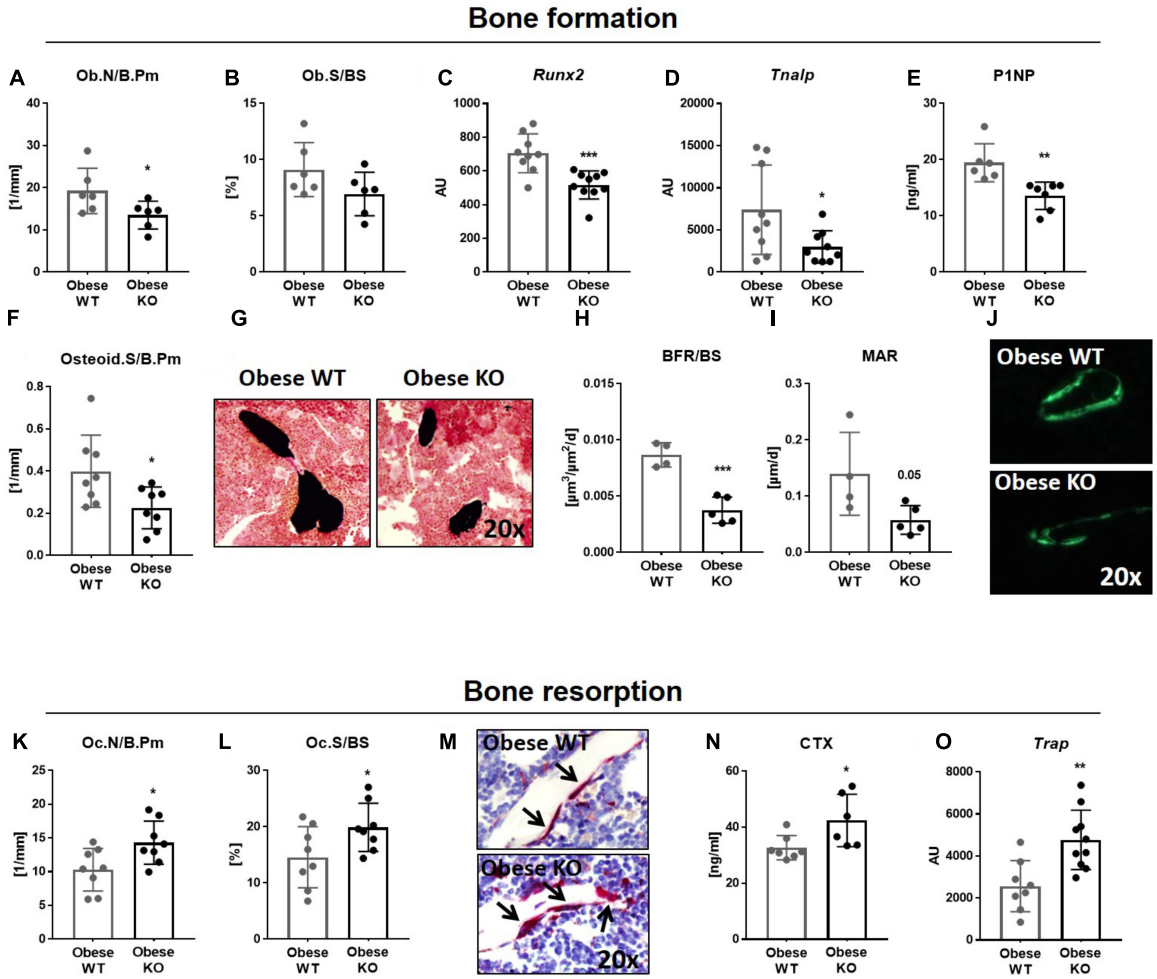

FIGURE 2 | Lack of Thy-1 reduces osteoblast differentiation as well as bone formation and increases osteoclast differentiation and bone resorption in obese mice. Wildtype (WT) and Thy-1$^{-/-}$ (KO) mice were fed with a high fat diet for 18 weeks (HFD). **(A–J)** Bone formation and (K-O) bone resorption were analyzed. The **(A)** osteoblast number per bone perimeter (Ob.N/B.Pm), **(B)** osteoblast surface per bone surface (Ob.S/BS) were analyzed using histology methods. Gene expression of the osteogenic markers **(C)** runt-related transcription factor 2 (*Runx2*), **(D)** and alkaline phosphatase (*Tnalp*) was analyzed by RT-PCR technique. **(E)** The serum concentration of total procollagen type 1 amino-terminal propeptide (P1NP) was analyzed using ELISA technique and the **(F)** osteoid surface per bone perimeter (Osteid.S/B.Pm) by histology. **(G)** Representative sections of von Kossa/van Gieson staining of bone (black) and cartilage (dense red area close to the bone). **(H)** The bone formation rate per bone surface (BFR/BS) as well as **(I)** mineral apposition rate (MAR) were determined by histomorphometric analysis (double calcein labeling). **(J)** Representative images of calcein labeling (green). **(K)** Osteoclast number per bone perimeter (Oc.N/B.Pm) and **(L)** osteoclast surface per bone surface (Oc.S/BS) were analyzed by staining of tartrate resistant acid phosphatase (TRAP). **(M)** Representative images of TRAP staining (red spots and black arrows = TRAP-positive cell/osteoclast). **(N)** Serum concentration of the bone resorption marker carboxy-terminal collagen crosslinks (CTX) was measured by ELISA technique. **(O)** Gene expression of *Trap* was evaluated via RT-PCR. Each point represents one mouse and median ± SD is presented. Asterisks denote significance level of $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test).

of osteoclast activity (+86%, **Figure 2O**), in bone of obese Thy-1$^{-/-}$ mice.

Lack of Thy-1 Promotes Obesity Mediated Inflammation

There is accumulating evidence that excessive adipose tissue accumulation in obesity is detrimental to bone health. Indeed, Thy-1$^{-/-}$ mice gained more weight compared to controls after HFD (Woeller et al., 2015; Paine et al., 2018; Picke et al., 2018a; **Figure 1A**). In addition to an increased adipocyte number (N.Adipo, +2.5-fold, **Figure 3A**), we observed an elevated adipocyte area (Adipo.Ar, +4-fold%, **Figure 3B**) as well as an upregulated gene expression of the fat marker *Fabp* in bone of obese Thy-1$^{-/-}$ mice (+1-fold, **Figure 3C**). Altogether an increased total fat volume (FV/TV, +56%, **Figures 3D,E**) was observed indicating expanded bone marrow adiposity in Thy-1$^{-/-}$ mice. Increased body fat mass is associated with latent inflammatory response (Greco et al., 2015). Consistent with this, the gene expression of the potent pro-inflammatory cytokines *Tnfα* and *Il6* (+130 and +46%, respectively, **Figures 3F,G**) were elevated in obese Thy-1$^{-/-}$ mice. Indeed, TNFα strongly increased osteoclast differentiation

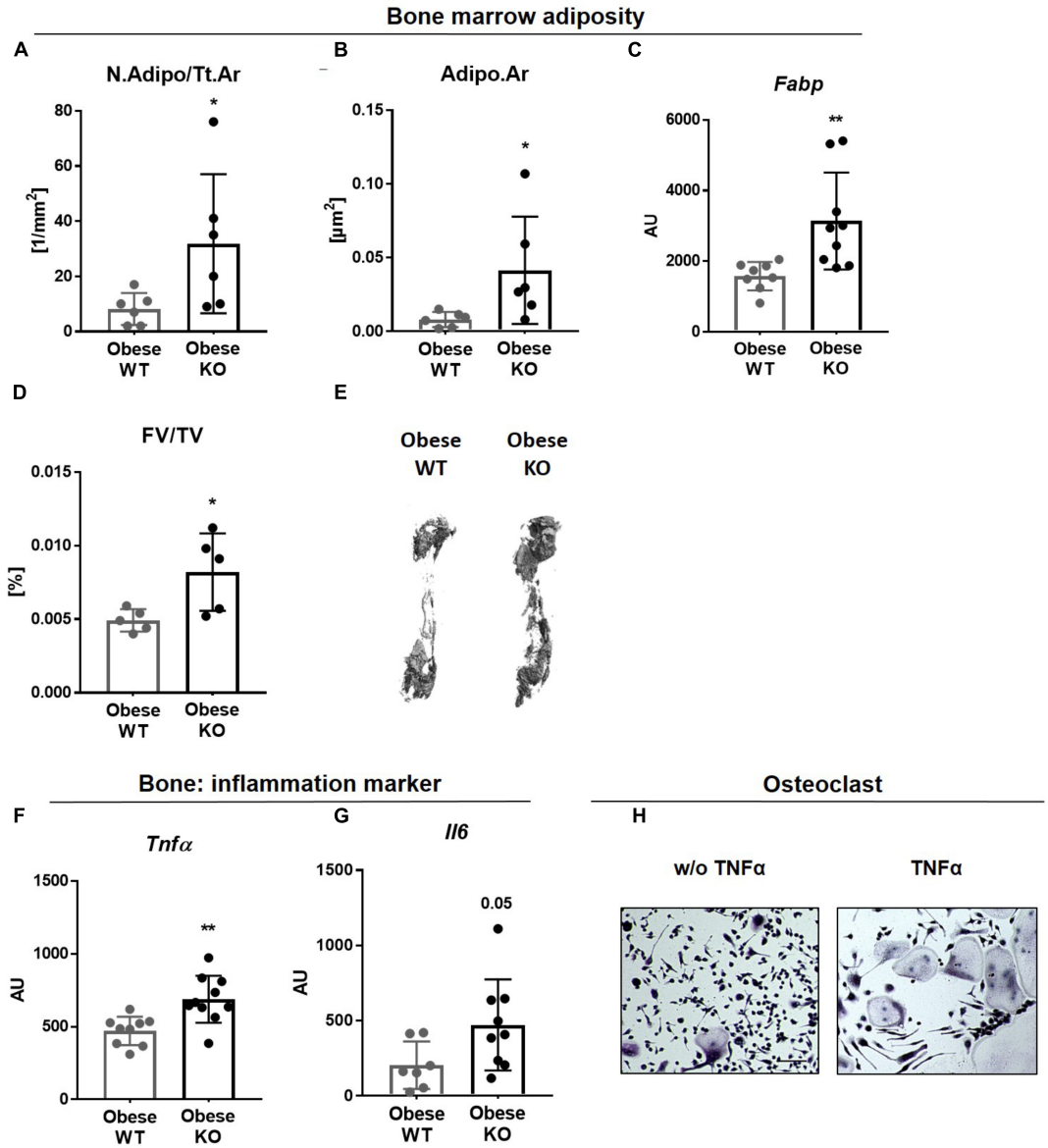

FIGURE 3 | Lack of Thy-1 promotes obesity mediated inflammation. Wildtype (WT) and Thy-1$^{-/-}$ (KO) mice were fed with a high fat diet for 18 weeks (HFD). **(A)** Number of adipocytes per total area (N.Adipo/Tt.Ar) and **(B)** adipocyte area (Adipo.Ar) of adipocytes were analyzed by histology technique. **(C)** Gene expression of the fat marker fatty-acid-binding protein (*Fabp*) in bone was assessed by RT-PCR. **(D)** Fat volume (FV/TV) in the femoral medullary cavity was analyzed by osmium tetroxide staining. **(E)** Representative 3D-images of the fat volume of whole femur. **(F,G)** Gene expression of the pro-inflammatory markers tumor necrosis factor α (*Tnfα*) and interleukin 6 (*Il6*). **(H)** Osteoclast precursor cells from WT mice were cultured *ex vivo* without (w/o) and with TNFα and osteoclastogenesis was detected via staining for tartrate resistant acid phosphatase (TRAP; giant, multinucleated, red cells = osteoclasts; indicated by arrows). Asterisks denote significance level of $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

shown by an increased number of multinucleated, TRAP positive giant cells (**Figure 3H**).

Thy-1$^{-/-}$ in Obesity Does Not Alter the Wnt and YAZ/TAZ Pathway

Osteogenic differentiation and bone formation is strongly regulated by Wnt signaling that is controlled by inhibitor molecules such as Dkk-1 and sclerostin (Pinzone et al., 2009; Kim et al., 2013). However, gene expression of *Dkk-1* and sclerostin (*Sost*, **Figures 4A,B**) as well as their serum concentrations (**Figures 4C,D**) were unaffected in obese Thy-1$^{-/-}$ mice. In line with these observations, the gene expression of non-canonical, *Wnt5a* and *Wnt11*, as well as canonical Wnt ligands, *Wnt3a* and *Wnt10b*, were not altered in bone by Thy-1 deficiency after HFD (**Figures 4E–H**). The Hippo signaling increases osteoblastogenesis (Pan et al., 2018). Neither

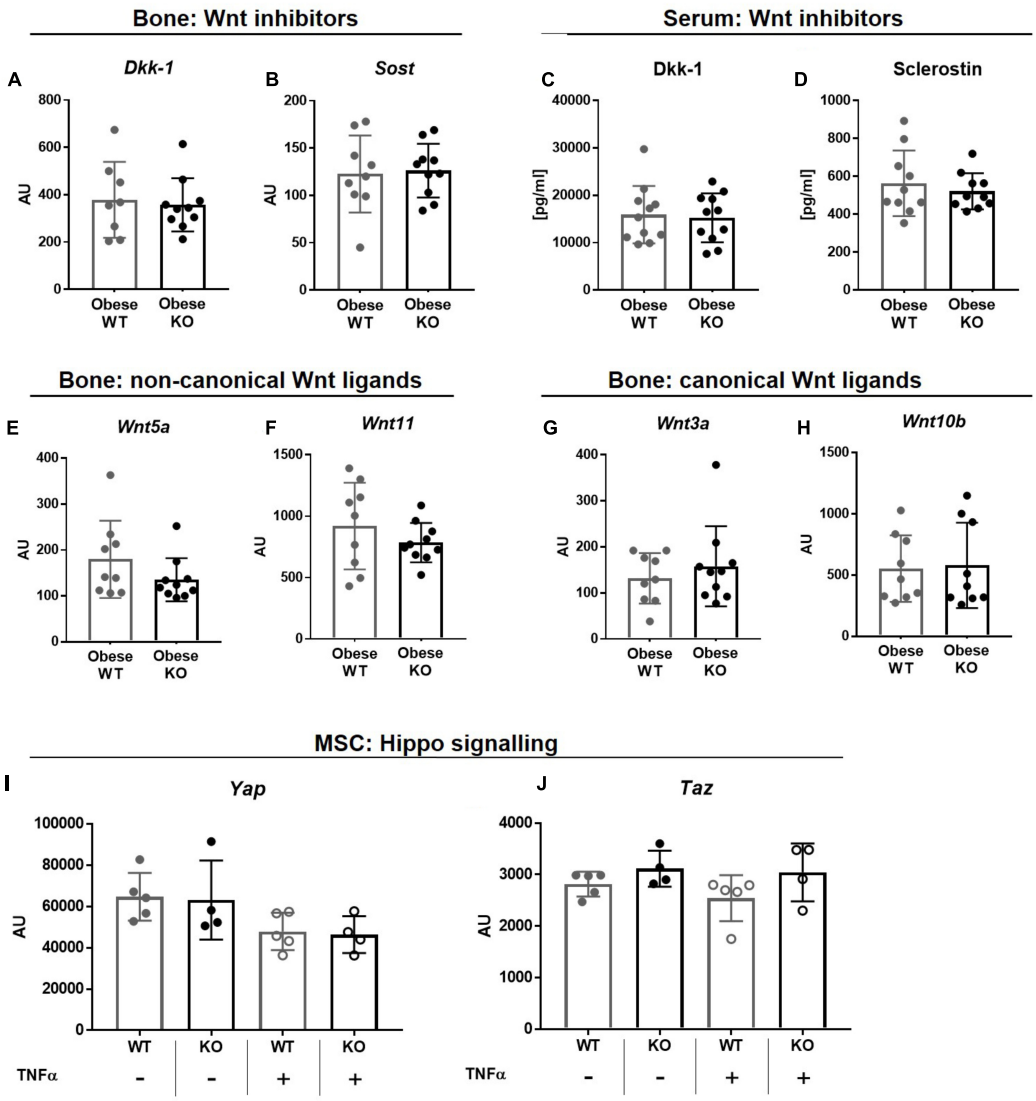

FIGURE 4 | Thy-$1^{-/-}$ in obesity does not alter the Wnt and YAZ/TAZ pathway. Wildtype (WT) and Thy-$1^{-/-}$ (KO) mice were fed with a high fat diet for a time period of 18 weeks (HFD). Gene expression of the Wnt pathway inhibitors **(A)** dickkopf-1 (*Dkk-1*) and **(B)** sclerostin (*Sost*) and **(C,D)** their serum concentrations were evaluated by RT-PCR and ELISA technique, respectively. Gene expression of Wnt ligands such as **(E)** *Wnt5a*, **(F)** *11*, **(G)** *3a*, and **(H)** *10b* in bone was analyzed by RT-PCR. MSCs from WT and Thy-$1^{-/-}$ mice were treated with TNFα and expression of *Yap* and *Taz* of the hippo signaling were investigated. Statistical analysis was performed by **(A–H)** Student's *t*-test and by **(I,J)** 2-way ANOVA.

Thy-1 deficiency nor treatment with TNFα altered gene expression of YAP or TAZ in wildtype and Thy-$1^{-/-}$ MSCs (**Figures 4I,J**).

In summary, Thy-1 does not alter bone formation by modulation of the Wnt pathway nor the Hippo signaling in Thy-$1^{-/-}$ mice.

Lack of Thy-1 in Obese Mice Alters the Gene Expression of RANKL, OPG, and CSF1 Under Inflammatory Conditions

Since the RANK-RANKL-OPG axis and binding of CSF1 to its receptor CSF1R, located at surface of osteoclasts, play central roles in osteoclastogenesis, we analyzed their gene expression in bone of WT and Thy-$1^{-/-}$ mice. The gene expression of *Tnfsf11,* which encodes for RANKL, was unaffected, while the expression of its decoy receptor *Tnfrsf11b,* encodes for OPG (p = 0.07) showed trend toward reduction in bone of obese Thy-$1^{-/-}$ mice (**Figures 5A,B**). The expression of *Csf1* was not detectable in bone, but the expression of its receptor *Csf1r* showed a trend toward upregulation (p = 0.07, **Figure 5C**). Obesity is characterized by latent inflammation shown by increased TNFα levels. Since RANKL, OPG, and CSF1 are expressed by stromal cells in bone, we stimulated WT and Thy-$1^{-/-}$ MSCs with TNFα to mimic the pro-inflammatory environment. Gene expression analysis revealed that Thy-1 does not affect

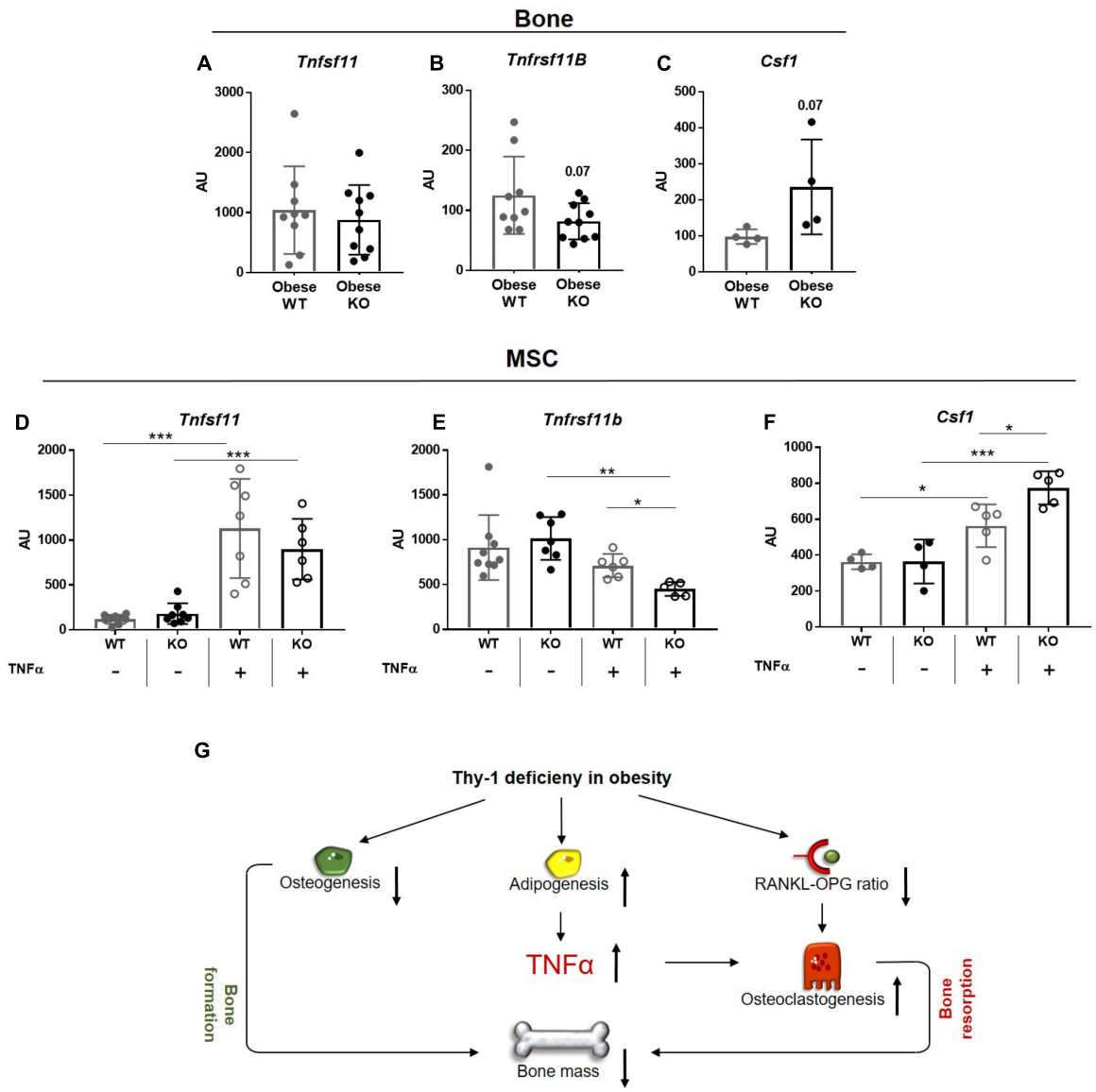

FIGURE 5 | Lack of Thy-1 in obese mice alters the gene expression of RANKL, OPG, and CSF1 under inflammatory conditions. Wildtype (WT) and Thy-$1^{-/-}$ (KO) mice were fed with a high fat diet for a time period of 18 weeks (HFD). **(A)** Gene expression of receptor activator of NF-κB ligand (RANKL, *Tnfsf11*), **(B)** its decoy receptor osteoprotegerin (OPG, *Tnfrsf11b*), and **(C)** receptor of Csf1 *(Cfs1r)* was analyzed in bone. MSCs from WT and KO mice were treated with TNFα for 24 h to mimic an inflammatory environment and gene expression of *Tnfsf11*, *Tnfrsf11b*, and *Cfs1* was determined **(D–F)**. **(G)** Summary figure of the key findings. In mice, Thy-1 deficiency results in a reduced osteoclastogenesis and increased adipogenesis leading to a decreased bone formation. Adipocytes produce more of the pro-inflammatory cytokine TNFα and the RANKL-OPG ratio is reduced resulting in an elevated osteoclastogenesis and poor bone mass. Each point represents one mouse and median ± SD is presented. Asterisks denote significance level of $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ measured by **(A–C)** Student's *t*-test and by **(D–F)** 2-way ANOVA.

the expression of *Tnfsf11*, *Tnfrsf11b*, and *Csf1* under basal conditions (**Figures 5D–F**). Upon pro-inflammatory stimulation, Thy-$1^{-/-}$ MSCs expressed reduced levels of *Tnfrs11b* (−36%, **Figure 5E**) while *Csf1* (+37%, **Figure 5F**) expression was elevated.

Taken together, Thy-1 leads to an unaltered *Tnfsf11,* reduced *Tnfrsf11b,* and increased *Csf1* expression in an inflammatory environment that might contribute to increased osteoclastogenesis in Thy-$1^{-/-}$ mice.

DISCUSSION

Alterations in bone mass and quality caused by osteoporosis and obesity are major health problems worldwide. They are caused by an unbalanced differentiation capacity and activity of bone-forming osteoblasts and bone-resorbing osteoclasts. The underlying mechanisms are still not fully understood and therefore sufficient therapy options are limited. One step toward identifying these mechanisms was the recent discovery that

the cell-surface protein Thy-1 is a major modulator of MSC differentiation into the adipogenic or osteogenic lineage (Hosoya et al., 2012; Chung et al., 2013; Picke et al., 2018a). In lean mice, Thy-1 deficiency increases whole body adipogenesis while decreasing osteoblast differentiation resulting in poor bone mass and quality indicating Thy-1 as a protector of bone mass (Woeller et al., 2015; Picke et al., 2018a). Therein, the activity of osteoclasts was not altered. Here, we went one step further and analyzed Thy-1 effects on bone under obese conditions. Thy-$1^{-/-}$ and WT mice fed a HFD developed an obese phenotype whereas Thy-1 deficiency exacerbated fat accumulation. Most importantly, Thy-1 deficiency decreased the bone mass under obese conditions by affecting both bone formation and bone resorption.

Thy-1 has been shown to promote osteogenesis and thereby inhibit adipogenesis (Hosoya et al., 2012; Chung et al., 2013; Woeller et al., 2015; Picke et al., 2018a). More specifically, inhibition of Thy-1 expression in MSCs isolated from different sources resulted in increased adipogenic differentiation mirrored by enhanced lipid droplet accumulation (Moraes et al., 2016). Moreover, ectopic expression of Thy-1 in adipocyte-like 3T3 cells inhibits adipogenic differentiation while depletion of endogenous Thy-1 in human fibroblasts increases their ability to undergo adipogenesis (Woeller et al., 2015; Picke et al., 2018a). Correspondingly, Thy-$1^{-/-}$ mice show an increased weight gain and body fat mass caused by inhibiting the activity of the Fyn kinase resulting in a reduced expression of PPARγ (Woeller et al., 2015; Picke et al., 2018a). On the other hand, Thy-1-positive MSCs, adipose-derived stromal cells and dental pulp cells show an increased ALP activity and mineralization capacity *in vitro* (Hosoya et al., 2012; Chung et al., 2013; Picke et al., 2018a). Upon subcutaneous injection, these cells increase formation of bone-like matrix and improve the healing of critical size defects (Hosoya et al., 2012; Chung et al., 2013). In contrast, Moraes et al. (2016) demonstrated that Thy-1 downregulation supports osteogenic differentiation of dental pulp cells and MSCs but, however, detected concurrently an increased adipogenic differentiation of cells with decreased Thy-1 expression. Importantly, we recently demonstrated that Thy-$1^{-/-}$ mice have a massive reduction of bone mass and quality independent of the gender when fed a standard chow (Picke et al., 2018a).

In the present study, we demonstrated that Thy-1 deficiency augmented obesity-mediated bone loss. In agreement with Paine et al. (2018) we found a reduced femoral bone volume and microstructure due to a decreased trabecular number and increased trabecular separation. Interestingly, bone mass of vertebral bodies was not altered in lean Thy-$1^{-/-}$ mice, but reduced in obese Thy-$1^{-/-}$ mice. This is an interesting finding as other studies also report varying effects on femur and vertebral bodies in rodent experiments. The negative effect of osteoporosis induced by ovariectomy on bone mass was found to be more pronounced in femur neck compared to the spine (Jiang et al., 1997). One reason could be that the spine of quadrupeds is in a horizontal position and could be therefore exposed to less mechanical loading in comparison to the femur (Smit, 2002). The increased mechanical loading brought about by weight gain in lean Thy-$1^{-/-}$ mice may not have been sufficient to affect trabecular bone mass at sites other than the femur.

In lean Thy-$1^{-/-}$ mice, the cortical bone mass and its biomechanical properties were significantly reduced compared to WT controls (Picke et al., 2018a). In contrast, HFD and Thy-1 deficiency did not alter the cortical bone mass. Consequently, bone quality markers such as cortical porosity and the moment of inertia were not affected by Thy-1 deficiency under obese conditions. Thy-1-mediated effects on cortical bone therefore seem to be compensated by as yet unknown mechanisms in obesity. In addition, in lean mice, Thy-1 deficiency was accompanied by increased expression of Wnt inhibitors, reduced expression of Wnt ligands and reduced responsiveness to Wnt stimulation (Picke et al., 2018a). However, obesity seems to overwrite these Thy-1-mediated effects on the Wnt pathway. Further, osteo-anabolic Hippo signaling was also not altered by neither Thy-1 deficiency nor mimicked the pro-inflammatory environment *ex vivo*.

Bone remodeling is a result of the balanced bone formation by bone-forming osteoblasts and bone resorption by bone-resorbing osteoclasts (Crockett et al., 2011). As shown in lean and obese mice Thy-1 deficiency reduced the number and activity of osteoblasts and thus impaired bone formation (Paine et al., 2018; Picke et al., 2018a). Interestingly, in lean mice, lack of Thy-1 neither affected number nor activity of osteoclasts. In contrast, upon 18 weeks of HFD Thy-1 deficiency enhanced the number and activity of osteoclasts and, thus, might force bone resorption. This might contribute to bone loss in obese Thy-1 deficient mice. Interestingly, Paine et al. (2018) described a reduction of osteoblasts in obese Thy-$1^{-/-}$ mice while osteoclasts were unaffected. However, Paine et al. (2018) analyzed mice upon 8 weeks of HFD. In our study, Thy-1 deficiency induced an enhanced weight gain from 12 weeks of HFD. Thus, Thy-1 deficiency impaired osteoblast differentiation and activity under both lean (Picke et al., 2018a) body composition and in the early and late phase of obesity. In contrast, an impact of Thy-1 deficiency on osteoclasts was only seen in late, distinct obesity.

Obesity is associated with latent inflammatory responses indicated by an elevated expression of pro-inflammatory cytokines such as Il1, Il6, and TNFα (Cao, 2011). Indeed, we detected an increased bone marrow adiposity in obese Thy-$1^{-/-}$ mice. Correspondingly, gene expression of *Il6* and *Tnf*α was up-regulated in the bone of obese Thy-$1^{-/-}$ compared to obese WT mice. IL-6 can be produced by osteoblasts and induces bone resorption (Ishimi et al., 1990). TNFα expression has been shown to be increased in obesity, arthritis, and osteoporosis. TNFα is able to affect both osteoclast differentiation and osteogenesis. TNFα produced by adipocytes potently reduces the differentiation of osteoblasts via TNFR1 or via upregulation of autophagy and reduction of apoptosis (Gilbert et al., 2000, 2005; Zhao et al., 2011; Abuna et al., 2016; Zheng et al., 2017). In obesity, arthritis, and osteoporosis, which are diseases that are characterized by a reduction of bone mass due to an increased osteoclastogenesis (Cao, 2011; Halade et al., 2011; Shu et al., 2015). TNFα has the ability to increase osteoclast differentiation by elevating the expression of RANKL, a master modulator

of osteoclastogenesis, by osteoblasts via TNFR1 and/or by the Pi3K/Akt pathway (Zhang et al., 2001; Siggelkow et al., 2003; Kitaura et al., 2013; Osta et al., 2014; Wu et al., 2017). TNFα alone does not increase the differentiation of osteoclast precursor cells (Kobayashi et al., 2000; Lam et al., 2000), but has been shown to act synergistically together with RANKL on osteoclastogenesis (Azuma et al., 2000; Lam et al., 2000; Fuller et al., 2002). In addition, TNFα can also elevate osteoclast differentiation when CSF1 is present (Kobayashi et al., 2000; Kitaura et al., 2005). *Ex vitro*, we demonstrated the augmentation of osteoclastogenesis by TNFα in the presence of RANKL and CSF1. Thus, despite of similar expression of *Tnfsf11* (RANKL) in bone of obese WT and Thy-1-deficient mice elevated amounts of TNFα in Thy-1-deficient mice might contribute via the synergistic action with RANKL to increased osteoclast number and activity in Thy-1 deficient mice.

In addition to the RANK-RANKL-OPG axis, the presence of CSF1 is essential for osteoclast differentiation (Kim and Kim, 2016). The reduced expression of RANKL decoy receptor, *Tnfsf11b* (OPG) and increased expression of *Csf1* under pro-inflammatory conditions in Thy-1$^{-/-}$ MSC might contribute to the increased osteoclastogenesis in obese Thy-1$^{-/-}$ mice.

However, one limitation of our study is that we could not demonstrate a direct relation *in vivo* between alteration of TNFα, CSF1, and OPG expression in obese Thy-1$^{-/-}$ mice and increased osteoclastogenesis and diminished bone mass. Because we were working with a global knockout mouse model, we cannot fully exclude effects of other cell types expressing Thy-1 on bone metabolism. Nevertheless, we showed previously that isolated MSCs from lean Thy-1$^{-/-}$ mice created less mineralized matrix in a WT environment *in vivo* (Picke et al., 2018a). However, we failed to discover the underlying mechanism of reduced osteoblastogenesis in Thy-1 deficiency in obese mice. It is known that obese patients have an increased concentration of unsaturated fatty acids that negatively affect osteoblastogenesis (Hardouin et al., 2016). In future, it should be addressed if this could also affect differentiation and function of Thy-1$^{-/-}$ MSCs.

Taken together, Thy-1 controls the balance between bone formation and bone resorption. In lean mice and in the early phase of obesity, a lack of Thy-1 impairs bone formation by inhibition of osteoblast differentiation, while there is no effect on bone resorption (Paine et al., 2018; Picke et al., 2018a). In the present study, we show that a lack of Thy-1 under manifest obese conditions affects both bone formation and bone resorption. Obese Thy-1$^{-/-}$ mice exhibited increased bone marrow adiposity associated with an increased pro-inflammatory environment including increased *Tnfa* expression. TNFα is a strong promotor of osteoclast differentiation and, thus, bone resorption. On the other hand lack of Thy-1 resulted in an attenuated expression of *Tnfsf11B* (OPG), the decoy receptor for *Tnfsf11* (RANKL). Both increased TNFα expression and diminished *Tnfsf11B* might induce bone resorption in obese Thy-1$^{-/-}$ mice (**Figure 5G**).

AUTHOR CONTRIBUTIONS

A-KP and AS designed the study, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. UA provided the experiences and methods for RNA analysis. MR and LH provided the skeletal expertise, methods for bone analysis, and interpreted the bone data. GC performed part of the μCT measurements and analyzed the data. FS and BB carried out the compositional analyses. JS discussed the data and revised the manuscript. All authors discussed the data and read and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00127/full#supplementary-material

REFERENCES

Abuna, R. P., De Oliveira, F. S., Santos T de, S., Guerra, T. R., Rosa, A. L., and Beloti, M. M. (2016). Participation of TNF-α in inhibitory effects of adipocytes on osteoblast differentiation. *J. Cell. Physiol.* 231, 204–214. doi: 10.1002/jcp.25073

Azuma, Y., Kaji, K., Katogi, R., Takeshita, S., and Kudo, A. (2000). Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. *J. Biol. Chem.* 275, 4858–4864. doi: 10.1074/jbc.275.7.4858

Boskey, A., and Pleshko Camacho, N. (2007). FT-IR imaging of native and tissue-engineered bone and cartilage. *Biomaterials* 28, 2465–2478. doi: 10.1016/j.biomaterials.2006.11.043

Boyce, B. F., and Xing, L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch. Biochem. Biophys.* 473, 139–146. doi: 10.1016/j.abb.2008.03.018

Cao, J. J. (2011). Effects of obesity on bone metabolism. *J. Orthop. Surg. Res.* 6:30. doi: 10.1186/1749-799X-6-30

Cao, J. J., Gregoire, B. R., and Gao, H. (2009). High-fat diet decreases cancellous bone mass but has no effect on cortical bone mass in the tibia in mice. *Bone* 44, 1097–1104. doi: 10.1016/j.bone.2009.02.017

Cao, J. J., Sun, L., and Gao, H. (2010). Diet-induced obesity alters bone remodeling leading to decreased femoral trabecular bone mass in mice. *Ann. N. Y. Acad. Sci.* 1192, 292–297. doi: 10.1111/j.1749-6632.2009.05252.x

Chung, M. T., Liu, C., Hyun, J. S., Lo, D. D., Montoro, D. T., Hasegawa, M., et al. (2013). CD90 (Thy-1)-positive selection enhances osteogenic capacity of human adipose-derived stromal cells. *Tissue Eng. Part A* 19, 989–997. doi: 10.1089/ten.tea.2012.0370

Cobayashi, F., Lopes, L. A., and Taddei, J. A. (2005). Bone mineral density in overweight and obese adolescents. *J. Pediatr.* 81, 337–342. doi: 10.2223/1372

Craig, W. (1993). Expression of Thy-1 on human hematopoietic progenitor cells. *J. Exp. Med.* 177, 1331–1342. doi: 10.1084/jem.177.5.1331

Crockett, J. C., Rogers, M. J., Coxon, F. P., Hocking, L. J., and Helfrich, M. H. (2011). Bone remodelling at a glance. *J. Cell Sci.* 124, 991–998. doi: 10.1242/jcs.063032

Fuller, K., Murphy, C., Kirstein, B., Fox, S. W., and Chambers, T. J. (2002). TNFalpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL. *Endocrinology* 143, 1108–1118. doi: 10.1210/endo.143.3.8701

Gilbert, L., He, X., Farmer, P., Boden, S., Kozlowski, M., Rubin, J., et al. (2000). Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* 141, 3956–3964. doi: 10.1210/endo.141.11.7739

Gilbert, L. C., Rubin, J., and Nanes, M. S. (2005). The p55 TNF receptor mediates TNF inhibition of osteoblast differentiation independently of apoptosis. *Am. J. Physiol. Metab.* 288, E1011–E1018. doi: 10.1152/ajpendo.00534.2004

Greco, E. A., Lenzi, A., and Migliaccio, S. (2015). The obesity of bone. *Ther. Adv. Endocrinol. Metab.* 6, 273–286. doi: 10.1177/2042018815611004

Halade, G. V., El Jamali, A., Williams, P. J., Fajardo, R. J., and Fernandes, G. (2011). Obesity-mediated inflammatory microenvironment stimulates osteoclastogenesis and bone loss in mice. *Exp. Gerontol.* 46, 43–52. doi: 10.1016/j.exger.2010.09.014

Hardcastle, S. A., Dieppe, P., Gregson, C. L., Arden, N. K., Spector, T. D., Hart, D. J., et al. (2015). Individuals with high bone mass have an increased prevalence of radiographic knee osteoarthritis. *Bone* 71, 171–179. doi: 10.1016/j.bone.2014.10.015

Hardouin, P., Rharass, T., and Lucas, S. (2016). Bone marrow adipose tissue: to be or not to be a typical adipose tissue? *Front. Endocrinol.* 7:85. doi: 10.3389/fendo.2016.00085

Hosoya, A., Hiraga, T., Ninomiya, T., Yukita, A., Yoshiba, K., Yoshiba, N., et al. (2012). Thy-1-positive cells in the subodontoblastic layer possess high potential to differentiate into hard tissue-forming cells. *Histochem. Cell Biol.* 137, 733–742. doi: 10.1007/s00418-012-0928-1

Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature* 444, 860–867. doi: 10.1038/nature05485

Hsu, Y. H., Venners, S. A., Terwedow, H. A., Feng, Y., Niu, T., Li, Z., et al. (2006). Relation of body composition, fat mass, and serum lipids to osteoporotic fractures and bone mineral density in Chinese men and women. *Am. J. Clin. Nutr.* 83, 146–154. doi: 10.1093/ajcn/83.1.146

Ishimi, Y., Miyaura, C., Jin, C. H., Akatsu, T., Abe, E., Nakamura, Y., et al. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J. Immunol.* 145, 3297–3303.

Jiang, Y., Zhao, J., Genant, H. K., Dequeker, J., and Geusens, P. (1997). Long-term changes in bone mineral and biomechanical properties of vertebrae and femur in aging, dietary calcium restricted, and/or estrogen-deprived/-replaced rats. *J. Bone Miner. Res.* 12, 820–831. doi: 10.1359/jbmr.1997.12.5.820

Kim, J. H., and Kim, N. (2016). Signaling pathways in osteoclast differentiation. *Chonnam Med. J.* 52, 12–17. doi: 10.4068/cmj.2016.52.1.12

Kim, J. H., Liu, X., Wang, J., Chen, X., Zhang, H., Kim, S. H., et al. (2013). Wnt signaling in bone formation and its therapeutic potential for bone diseases. *Ther. Adv. Musculoskelet. Dis.* 5, 13–31. doi: 10.1177/1759720X12466608

Kitaura, H., Kimura, K., Ishida, M., Kohara, H., Yoshimatsu, M., and Takano-Yamamoto, T. (2013). Immunological reaction in TNF- α -mediated osteoclast formation and bone resorption *In vitro* and *In vivo*. *Clin. Dev. Immunol.* 2013:181849. doi: 10.1155/2013/181849

Kitaura, H., Zhou, P., Kim, H.-J., Novack, D. V., Ross, F. P., and Teitelbaum, S. L. (2005). M-CSF mediates TNF-induced inflammatory osteolysis. *J. Clin. Invest.* 115, 3418–3427. doi: 10.1172/JCI26132

Kling, J. M., Clarke, B. L., and Sandhu, N. P. (2014). Osteoporosis prevention, screening, and treatment: a review. *J. Womens Health* 23, 563–572. doi: 10.1089/jwh.2013.4611

Kobayashi, K., Takahashi, N., Jimi, E., Udagawa, N., Takami, M., Kotake, S., et al. (2000). Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J. Exp. Med.* 191, 275–286. doi: 10.1084/jem.191.2.275

Lam, J., Takeshita, S., Barker, J. E., Kanagawa, O., Ross, F. P., and Teitelbaum, S. L. (2000). TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J. Clin. Invest.* 106, 1481–1488. doi: 10.1172/JCI11176

Moraes, D. A., Sibov, T. T., Pavon, L. F., Alvim, P. Q., Bonadio, R. S., Da Silva, J. R., et al. (2016). A reduction in CD90 (THY-1) expression results in increased differentiation of mesenchymal stromal cells. *Stem Cell Res. Ther.* 7:97. doi: 10.1186/s13287-016-0359-3

Nielson, C. M., Srikanth, P., and Orwoll, E. S. (2012). Obesity and fracture in men and women: an epidemiologic perspective. *J. Bone Miner. Res.* 27, 1–10. doi: 10.1002/jbmr.1486

Osta, B., Benedetti, G., and Miossec, P. (2014). Classical and paradoxical effects of TNF-α on bone homeostasis. *Front. Immunol.* 5:48. doi: 10.3389/fimmu.2014.00048

Paine, A., Woeller, C. F., Zhang, H., de la Luz Garcia-Hernandez, M., Huertas, N., Xing, L., et al. (2018). Thy1 is a positive regulator of osteoblast differentiation and modulates bone homeostasis in obese mice. *FASEB J.* 32, 3174–3183. doi: 10.1096/fj.201701379R

Palermo, A., Tuccinardi, D., Defeudis, G., Watanabe, M., D'Onofrio, L., Lauria, A. P., et al. (2016). BMI and BMD: the potential interplay between obesity and bone fragility. *Int. J. Environ. Res. Public Health* 13:E544. doi: 10.3390/ijerph13060544

Pan, J.-X., Xiong, L., Zhao, K., Zeng, P., Wang, B., Tang, F.-L., et al. (2018). YAP promotes osteogenesis and suppresses adipogenic differentiation by regulating β-catenin signaling. *Bone Res.* 6:18. doi: 10.1038/s41413-018-0018-7

Parfitt, A. M., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., et al. (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR histomorphometry nomenclature committee. *J. Bone Miner. Res.* 2, 595–610. doi: 10.1002/jbmr.5650020617

Patsch, J. M., Kiefer, F. W., Varga, P., Pail, P., Rauner, M., Stupphann, D., et al. (2011). Increased bone resorption and impaired bone microarchitecture in short-term and extended high-fat diet-induced obesity. *Metabolism* 60, 243–249. doi: 10.1016/j.metabol.2009.11.023

Picke, A.-K., Campbell, G. M., Blüher, M., Krügel, U., Schmidt, F. N., Tsourdi, E., et al. (2018a). Thy-1 (CD90) promotes bone formation and protects against obesity. *Sci. Transl. Med.* 10:eaao6806. doi: 10.1126/scitranslmed.aao6806

Picke, A.-K., Gordaliza Alaguero, I., Campbell, G. M., Glüer, C.-C., Salbach-Hirsch, J., Rauner, M., et al. (2016). Bone defect regeneration and cortical bone parameters of type 2 diabetic rats are improved by insulin therapy. *Bone* 82, 108–115. doi: 10.1016/j.bone.2015.06.001

Picke, A.-K., Sylow, L., Møller, L. L. V., Kjøbsted, R., Schmidt, F. N., Steejn, M. W., et al. (2018b). Differential effects of high-fat diet and exercise training on bone and energy metabolism. *Bone* 116, 120–134. doi: 10.1016/j.bone.2018.07.015

Pinzone, J. J., Hall, B. M., Thudi, N. K., Vonau, M., Qiang, Y.-W., Rosol, T. J., et al. (2009). The role of Dickkopf-1 in bone development, homeostasis, and disease. *Blood* 113, 517–525. doi: 10.1182/blood-2008-03-145169

Schmidt, M., Gutknecht, D., Simon, J. C., Schulz, J.-N., Eckes, B., Anderegg, U., et al. (2015). Controlling the balance of fibroblast proliferation and differentiation: impact of Thy-1. *J. Invest. Dermatol.* 135, 1893–1902. doi: 10.1038/jid.2015.86

Sharma, S., Tandon, V. R., Mahajan, S., Mahajan, V., and Mahajan, A. (2014). Obesity: friend or foe for osteoporosis. *J. Midlife Health* 5, 6–9. doi: 10.4103/0976-7800.127782

Shu, L., Beier, E., Sheu, T., Zhang, H., Zuscik, M., Puzas, E., et al. (2015). High-fat diet causes bone loss in young mice by promoting osteoclastogenesis through alteration of the bone marrow environment. *Calcif. Tissue Int.* 96, 313–323. doi: 10.1037/emo0000122.Do

Siggelkow, H., Eidner, T., Lehmann, G., Viereck, V., Raddatz, D., Munzel, U., et al. (2003). Cytokines, osteoprotegerin, and rankl in vitro and histomorphometric indices of bone turnover in patients with different bone diseases. *J. Bone Miner. Res.* 18, 529–538. doi: 10.1359/jbmr.2003.18.3.529

Smit, T. H. (2002). The use of a quadruped as an in vivo model for the study of the spine - biomechanical considerations. *Eur. Spine J.* 11, 137–144. doi: 10.1007/s005860100346

Thurner, P. J. (2009). Atomic force microscopy and indentation force measurement of bone. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 1, 624–649. doi: 10.1002/wnan.56

Tu, K. N., Lie, J. D., Wan, C. K. V., Cameron, M., Austel, A. G., Nguyen, J. K., et al. (2018). Osteoporosis: a review of treatment options. *P T* 43, 92–104.

Vitetta, E. S., Boyse, E. A., and Uhr, J. W. (1973). Isolation and characterization of a molecular complex containing thy-1 antigen from the surface of murine thymocytes and t cells. *Eur. J. Immunol.* 3, 446–453. doi: 10.1002/eji.1830030714

Wetzel, A., Chavakis, T., Preissner, K. T., Sticherling, M., Haustein, U.-F., Anderegg, U., et al. (2004). Human Thy-1 (CD90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J. Immunol.* 172, 3850–3859. doi: 10.4049/jimmunol.172.6.3850

Woeller, C. F., O'Loughlin, C. W., Pollock, S. J., Thatcher, T. H., Feldon, S. E., and Phipps, R. P. (2015). Thy1 (CD90) controls adipogenesis by regulating activity of the Src family kinase. Fyn. *FASEB J.* 29, 920–931. doi: 10.1096/fj.14-257121

Wu, L., Guo, Q., Yang, J., and Ni, B. (2017). Tumor necrosis factor alpha promotes osteoclast formation via PI3K/Akt pathway-mediated blimp1 expression upregulation. *J. Cell. Biochem.* 118, 1308–1315. doi: 10.1002/jcb.25672

Zhang, Y. H., Heulsmann, A., Tondravi, M. M., Mukherjee, A., and Abu-Amer, Y. (2001). Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J. Biol. Chem.* 276, 563–568. doi: 10.1074/jbc.M008198200

Zhao, L., Huang, J., Zhang, H., Wang, Y., Matesic, L. E., Takahata, M., et al. (2011). Tumor necrosis factor inhibits mesenchymal stem cell differentiation into osteoblasts via the ubiquitin E3 ligase Wwp1. *Stem Cells* 29, 1601–1610. doi: 10.1002/stem.703

Zhao, L. J., Liu, Y. J., Liu, P. Y., Hamilton, J., Recker, R. R., and Deng, H. W. (2007). Relationship of obesity with osteoporosis. *J. Clin. Endocrinol. Metab.* 92, 1640–1646. doi: 10.1210/jc.2006-0572

Zheng, L., Wang, W., Ni, J., Mao, X., Song, D., Liu, T., et al. (2017). Role of autophagy in tumor necrosis factor-α-induced apoptosis of osteoblast cells. *J. Investig. Med.* 65, 1014–1020. doi: 10.1136/jim-2017-000426

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OPINION
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Defining Skin Fibroblastic Cell Types Beyond CD90

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Fibroblasts are the primary mesenchyme cell types that provide structural support during organ development and growth, and are the primary depositors of connective tissue matrix in response to injuries such as those occurring during skin scarring, tissue/organ fibrosis, systemic sclerosis, abdominal adhesions, just to name a few. Major efforts to study fibroblastic characteristics have centered on identifying surface markers that allow fibroblast purifications from tissues/organs.

The glycoprotein CD90 is a widely expressed mesenchymal cell surface marker that is the subject of almost 1,000 publications (PubMed). It is present on mesenchymal stem cells (Dominici et al., 2006), fibroblasts of various organs (Kisselbach et al., 2009) and myofibroblasts (Saada et al., 2006), and in connective tissues throughout anatomic locations, including skin (Jahoda et al., 2003; Nazari et al., 2016), liver (Katsumata et al., 2017), heart (Nural-Guvener et al., 2014), eye (Khoo et al., 2008). CD90 is also found on mesenchyme within tumors that promote tumor growth (True et al., 2010). Based on CD90s expression on various mesenchyme cell types it has been considered as a defining fibroblastic marker (Katsumata et al., 2017).

Fibroblasts were originally described as a single cell type (Ramon y Cajal, 1900). However, recent studies by us and others have demonstrated that dermal fibroblasts are an assortment of phenotypically and functionally heterogeneous cells (Driskell et al., 2013; Rinkevich et al., 2015; Singhal et al., 2016; Jiang et al., 2018; Philippeos et al., 2018). The various dermal fibroblast subtypes have drastically diverged functions, during skin development, upon wounding and at homeostasis. These different dermal fibroblast cell types can be isolated based on unique gene expression or profiles of combination of surface markers such as CD26, Blimp1, Dlk1, Sca1 in mouse (Driskell et al., 2013; Rinkevich et al., 2015), and CD26, CD39, CD36, RGS5 in human (Philippeos et al., 2018). In addition, the α5 chain of collagen VI (COL6A5) is highly enriched in human papillary fibroblasts (Fitzgerald et al., 2008), and the discoidin-domain receptor 2 (DDR2) enriches for human cardiac fibroblasts (Goldsmith et al., 2004).

CD90 alone therefore is not an accurate marker to define fibroblasts in general or its subtypes, because of the following reasons:

First, CD90 cannot distinguish dermal mesenchymal stem cells from dermal fibroblasts (Haniffa et al., 2009; Chang et al., 2014). Although CD90 is used as a mesenchymal stem cell marker (Dominici et al., 2006), it is identically expressed on the more differentiated dermal fibroblasts (Halfon et al., 2011; Fang et al., 2017).

Second, CD90 does not discriminate between functionally unique fibroblast subtypes. Fibroblasts are functionally heterogeneous, both between and within anatomic skin locations. For example, the oral cavity skin quickly seals open wounds with minimal scarring, while back-skin responds to injuries with an opaque plug of dense connective tissue. Fibroblasts taken from various skin depths also diverge functionally (Sorrell and Caplan, 2004; Philippeos et al., 2018). Fibroblasts from the upper skin layer (papillary) are pro-regenerative, whereas the fibroblasts from the deeper skin layer (reticular) are pro-fibrotic (Driskell et al., 2013). In addition to the anatomic heterogeneity, our group recently identified two additional different fibroblasts lineages in mouse back-skin that can be distinguished based on their transient early embryonic expression of *Engrailed-1* (*En1*) gene. The two fibroblast lineages either do or do not have a past history of *En1* expression, and are referred to as "En1-Past" Fibroblasts (EPFs) and "En1-Naive" Fibroblasts (ENFs). Using genetic lineage tracing and live imaging tools, we demonstrated that EPFs are the primary contributors to the scar phenotype

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in the back in all injury models tested, including cutaneous wounding and irradiation-induced tissue fibrosis, whereas ENFs do not participate or contribute to scarring (Rinkevich et al., 2015). In early fetal wounds ENFs typically sculpt and regenerate the original connective-tissue foundation (Jiang et al., 2018). The relative proportion of the two fibroblastic lineages changes radically during skin development, from an ENF-predominant to an EPF-predominant dermis, which leads to the skin's phenotypic transition from regeneration to scarring (Jiang et al., 2018). The flow cytometric analysis of CD90 surface expression on back-skin fibroblasts from embryonic day 18.5 (E18.5) mouse embryos reveals that CD90 is expressed on most, but not all, cells of both the EPF and ENF lineages (Rinkevich et al., 2015). This indicates that CD90 does not accurately mark any functional fibroblast subtype, nor does CD90 discriminate between anatomically distinct fibroblasts. For example, CD90 is expressed by fibroblasts in all layers of dermis, including papillary dermis, reticular dermis and hypodermis (Driskell et al., 2013), as well as different anatomic sites with diverged scarring and regenerative outcomes such as oral cavity and back-skin.

Thirdly, by large scale screening of surface markers we found that CD90 does not mark a single defined skin fibroblast subtype, and this was recently confirmed by single-cell transcriptome analysis, in both human and mouse (Rinkevich et al., 2015; Singhal et al., 2016; Philippeos et al., 2018). Therefore, future charting fibroblast subpopulations cannot rely on markers like CD90 but rather rely on lineage tracing studies that determine the fibroblast subsets based on distinct cellular functions.

The functional role of CD90 on the surface of fibroblasts is not well understood, with just a handful of intimations. CD90 on cancer-associated fibroblasts has been shown to contribute to inflammation by promoting fibroblast release of IL-6, which promotes tumor progression (Shiga et al., 2015; Huynh et al., 2016). On dermal fibroblasts, recent studies suggest that CD90 functions to dampen the expressions of the adipogenic markers PPARγ, and the Src-family kinase Fyn (Woeller et al., 2015), thereby blocking the differentiation of mesenchymal cells into adipocytes. However, the conclusion was derived from the experimental setup with a preadipocyte cell line under adipogenic induction. A direct lineage relationship between fibroblasts and adipocytes has not been directly proven so far. The observation that primary human and mouse adipocytes do not express CD90 (Phipps et al., 2012; Woeller et al., 2015) may simply suggest that adipocytes diverge from fibroblasts prior to and regardless of CD90 expression. Interestingly, a recent study shows that CD90 coupled with integrin regulates Src-family kinases at focal adhesions. Thus, CD90 may play a role in fibrogenesis by contributing to environmental rigidity sensing (Fiore et al., 2015). However, it is not clear whether this is a unique function of CD90 or a common property of a family of structurally related proteins that can bind integrins to trigger their conformational changes and alter their signaling function.

The relationship of CD90 to fibrosis is indeed far from clear. The loss of CD90 from lung fibroblasts is observed in idiopathic pulmonary fibrosis (Sanders et al., 2008) and results in a more severe fibrotic outcome (Hagood et al., 2005). In contrast, CD90 expression positively correlates with fibrosis on dermal fibroblasts in systemic sclerosis (Nazari et al., 2016) and on liver fibroblasts in cholestatic liver injury (Katsumata et al., 2017). The expression of matrix metalloproteinase inhibitor Timp1 is found to be upregulated in $CD90^+$ fibroblasts near the portal vein, suggesting these cells inhibit collagen degradation and promote accumulation of extracellular matrix (Katsumata et al., 2017). In line with these observations, CD90 expression is elevated in the capsular contracture and scar tissue after the implant-based breast reconstruction, and CD90 expression is essential for the myofibroblast phenotype in capsular fibroblasts (Hansen et al., 2017). In addition, higher CD90 expression is found to be accompanied by the increment of α-SMA^+ stromal component in hepatocellular carcinoma (Sukowati et al., 2013), implying for fibrotic and tumor promoting functions of CD90.

Defining fibroblast subsets based on their specific function could pave the way for clinical applications, and defining surface markers that mark specific classes of fibroblasts is a first step toward their cell enrichment. For examples, we have learned from previous studies that upper dermis fibroblasts promote the hair follicle regeneration, whereas lower dermis fibroblasts participate in wound healing (Driskell et al., 2013; Rognoni et al., 2016). Transplanted ENFs reduce skin scarring (Jiang et al., 2018). The functions of the specific fibroblast subsets are derived from animal studies, and the human homologous requires further investigation. The shrouded functions of CD90 on fibroblasts may arise from the heterogeneous compositions of fibroblasts in tissues. In which, CD90 relays multiple or opposing functions in distinct fibroblast lineages. The fibroblast lineage-specific functions of CD90 await future investigations.

We anticipate that fibroblasts exhibit a panoply of functionally distinct cell types, rivaling that of the hematopoietic system – different subsets carry different physiological functions and may display distinguishing surface markers. While the marker definition for hematopoietic subsets has been well established, the identification of markers for the various fibroblast subsets has just begun. The accumulating single cell transcriptome data on stromal cells from various tissues/organs including skin, may shed light on the unique markers for fibroblast subsets, and would pave the way toward cell enrichment strategies for clinical use.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Chang, Y., Li, H., and Guo, Z. (2014). Mesenchymal stem cell-like properties in fibroblasts. *Cell Physiol Biochem*. 34, 703–714. doi: 10.1159/000363035

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317. doi: 10.1080/14653240600855905

Driskell, R. R., Lichtenberger, B. M., Hoste, E., Kretzschmar, K., Simons, B. D., Charalambous, M., et al. (2013). Distinct fibroblast lineages determine dermal architecture in skin development and repair. *Nature* 504, 277–281. doi: 10.1038/nature12783

Fang, F., Ni, K., Cai, Y., Ye, Z., Shang, J., Shen, S., et al. (2017). Biological characters of human dermal fibroblasts derived from foreskin of male infertile patients. *Tissue Cell*. 49, 56–63. doi: 10.1016/j.tice.2016.12.003

Fiore, V. F., Strane, P. W., Bryksin, A. V., White, E. S., Hagood, J. S., and Barker, T. H. (2015). Conformational coupling of integrin and Thy-1 regulates Fyn priming and fibroblast mechanotransduction. *J. Cell Biol*. 211, 173–190. doi: 10.1083/jcb.201505007

Fitzgerald, J., Rich, C., Zhou, F. H., and Hansen, U. (2008). Three novel collagen VI chains, alpha4(VI), α5(VI), and α6(VI). *J. Biol. Chem*. 283, 20170–20180. doi: 10.1074/jbc.M710139200

Goldsmith, E. C., Hoffman, A., Morales, M. O., Potts, J. D., Price, R. L., McFadden, A., et al. (2004). Organization of fibroblasts in the heart. *Dev. Dyn*. 230, 787–794. doi: 10.1002/dvdy.20095

Hagood, J. S., Prabhakaran, P., Kumbla, P., Salazar, L., MacEwen, M. W., Barker, T. H., et al. (2005). Loss of fibroblast Thy-1 expression correlates with lung fibrogenesis. *Am. J. Pathol*. 167, 365–379. doi: 10.1016/S0002-9440(10)62982-3

Halfon, S., Abramov, N., Grinblat, B., and Ginis, I. (2011). Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging. *Stem Cells Dev*. 20, 53–66. doi: 10.1089/scd.2010.0040

Haniffa, M. A., Collin, M. P., Buckley, C. D., and Dazzi, F. (2009). Mesenchymal stem cells: the fibroblasts' new clothes? *Haematologica* 94, 258–263. doi: 10.3324/haematol.13699

Hansen, T. C., Woeller, C. F., Lacy, S. H., Koltz, P. F., Langstein, H. N., and Phipps, R. P. (2017). Thy1 (CD90) expression is elevated in radiation-induced periprosthetic capsular contracture: implication for novel therapeutics. *Plast. Reconstr. Surg*. 140, 316–326. doi: 10.1097/PRS.0000000000003542

Huynh, P. T., Beswick, E. J., Coronado, Y. A., Johnson, P., O'Connell, M. R., Watts, T., et al. (2016). $CD90^+$ stromal cells are the major source of IL-6, which supports cancer stem-like cells and inflammation in colorectal cancer. *Int. J. Cancer* 138, 1971–1981. doi: 10.1002/ijc.29939

Jahoda, C. A., Whitehouse, J., Reynolds, A. J., and Hole, N. (2003). Hair follicle dermal cells differentiate into adipogenic and osteogenic lineages. *Exp. Dermatol*. 12, 849–859. doi: 10.1111/j.0906-6705.2003.00161.x

Jiang, D., Correa-Gallegos, D., Christ, S., Stefanska, A., Liu, J., Ramesh, P., et al. (2018). Two succeeding fibroblastic lineages drive dermal development and the transition from regeneration to scarring. *Nat. Cell Biol*. 20, 422–431. doi: 10.1038/s41556-018-0073-8

Katsumata, L. W., Miyajima, A., and Itoh, T. (2017). Portal fibroblasts marked by the surface antigen Thy1 contribute to fibrosis in mouse models of cholestatic liver injury. *Hepatol Commun*. 1, 198–214. doi: 10.1002/hep4.1023

Khoo, T. K., Coenen, M. J., Schiefer, A. R., Kumar, S., and Bahn, R. S. (2008). Evidence for enhanced Thy-1 (CD90) expression in orbital fibroblasts of patients with Graves' ophthalmopathy. *Thyroid* 18, 1291–1296. doi: 10.1089/thy.2008.0255

Kisselbach, L., Merges, M., Bossie, A., and Boyd, A. (2009). CD90 Expression on human primary cells and elimination of contaminating fibroblasts from cell cultures. *Cytotechnology* 59, 31–44. doi: 10.1007/s10616-009-9190-3

Nazari, B., Rice, L. M., Stifano, G., Barron, A. M., Wang, Y. M., Korndorf, T., et al. (2016). Altered Dermal Fibroblasts in Systemic Sclerosis Display Podoplanin and CD90. *Am. J. Pathol*. 186, 2650–2664. doi: 10.1016/j.ajpath.2016.06.020

Nural-Guvener, H. F., Zakharova, L., Nimlos, J., Popovic, S., Mastroeni, D., Gaballa, M. A., et al. (2014). HDAC class I inhibitor, Mocetinostat, reverses cardiac fibrosis in heart failure and diminishes $CD90^+$ cardiac myofibroblast activation. *Fibrogenesis Tissue Repair* 7:10. doi: 10.1186/1755-1536-7-10

Philippeos, C., Telerman, S. B., Oulès, B., Pisco, A. O., Shaw, T. J., Elgueta, R., et al. (2018). Spatial and single-cell transcriptional profiling identifies functionally distinct human dermal fibroblast subpopulations. *J. Invest. Dermatol*. 138, 811–825. doi: 10.1016/j.jid.2018.01.016

Phipps, R. P., and Woeller, C. F., Feldon, S. E. (2012). *THY1 (CD90) As a Novel Therapy to Control Adipose Tissue Accumulation*. U.S. Patent No: US9694050B2. Rochester, NY: University of Rochester.

Ramon y Cajal, S. (1900). *Manual de Anatomía Patológica General*. Madrid: Imp. y Libreria de Nicolas Moya, 3rd Edn.

Rinkevich, Y., Walmsley, G. G., Hu, M. S., Maan, Z. N., Newman, A. M., Drukker, M., et al. (2015). Skin fibrosis. Identification and isolation of a dermal lineage with intrinsic fibrogenic potential. *Science* 348:aaa2151. doi: 10.1126/science.aaa2151

Rognoni, E., Gomez, C., Pisco, A. O., Rawlins, E. L., Simons, B. D., Watt, F. M., et al. (2016). Inhibition of β-catenin signalling in dermal fibroblasts enhances hair follicle regeneration during wound healing. *Development* 143, 2522–2535. doi: 10.1242/dev.131797

Saada, J. I., Pinchuk, I. V., Barrera, C. A., Adegboyega, P. A., Suarez, G., Mifflin, R. C., et al. (2006). Subepithelial myofibroblasts are novel nonprofessional APCs in the human colonic mucosa. *J. Immunol*. 177, 5968–5979. doi: 10.4049/jimmunol.177.9.5968

Sanders, Y. Y., Pardo, A., Selman, M., Nuovo, G. J., Tollefsbol, T. O., Siegal, G. P., et al. (2008). Thy-1 promoter hypermethylation: a novel epigenetic pathogenic mechanism in pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol*. 39, 610–618. doi: 10.1165/rcmb.2007-0322OC

Shiga, K., Hara, M., Nagasaki, T., Sato, T., Takahashi, H., and Takeyama, H. (2015). Cancer-associated fibroblasts: their characteristics and their roles in tumor growth. *Cancers* 7, 2443–2458. doi: 10.3390/cancers7040902

Singhal, P. K., Sassi, S., Lan, L., Au, P., Halvorsen, S. C., Fukumura, D., et al. (2016). Mouse embryonic fibroblasts exhibit extensive developmental and phenotypic diversity. *Proc. Natl. Acad. Sci. U.S.A*. 113, 122–127. doi: 10.1073/pnas.1522401112

Sorrell, J. M., and Caplan, A. I. (2004). Fibroblast heterogeneity: more than skin deep. *J. Cell Sci*. 117, 667–675. doi: 10.1242/jcs.01005

Sukowati, C. H., Anfuso, B., Torre, G., Francalanci, P., Croc,è, L. S., and Tiribelli, C. (2013). The expression of CD90/Thy-1 in hepatocellular carcinoma: an *in vivo* and *in vitro* study. *PLoS ONE* 8:e76830. doi: 10.1371/journal.pone.0076830

True, L. D., Zhang, H., Ye, M., Huang, C. Y., Nelson, P. S., von Haller, P. D., et al. (2010). CD90/THY1 is overexpressed in prostate cancer-associated fibroblasts and could serve as a cancer biomarker. *Mod. Pathol*. 23, 1346–1356. doi: 10.1038/modpathol.2010.122

Woeller, C. F., O'Loughlin, C. W., Pollock,. S. J., Thatcher, T. H., Feldon, S. E., and Phipps, R. P. (2015). Thy1 (CD90) controls adipogenesis by regulating activity of the Src family kinase, Fyn. *FASEB J*. 29, 920–931. doi: 10.1096/fj.14-257121

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MINI REVIEW
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Thy-1 as an Integrator of Diverse Extracellular Signals

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Thy-1 was discovered over 50 years ago, and in that time investigators from a broad variety of fields have described numerous and heterogeneous biological functions of Thy-1 in multiple contexts. As an outwardly facing cell surface molecule, it is well positioned to receive extracellular signals; previously reviewed studies have confirmed an important role in cell-cell and cell-matrix adhesion, cell migration, and regulation of outside-in signaling. More recent studies reviewed here expand the repertoire of Thy-1 effects on signaling pathways, and reveal novel roles in mechanotransduction, cellular differentiation, viral entry, and extracellular vesicle binding and internalization. All of these studies contribute to understanding Thy-1 as a context-dependent integrator of a diverse range of extracellular information, and provide impetus for further studies, some of which are suggested here.

Keywords: Thy-1, signaling, mechanotransduction, stem cells, viral entry, extracellular vesicles

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INTRODUCTION

More than 50 years after its original description as a lymphocyte marker (Reif and Allen, 1964), Thy-1 (CD90) remains an enigmatic molecule. It is expressed on the surface of numerous and diverse cell types, and confers varied effects on cell phenotype, depending on context (reviewed in Bradley et al., 2009). Accordingly, the functions of Thy-1 have been studied in a broad range of fields, including immunology, neurobiology, cancer, stem cell biology, tissue remodeling and aging, and have been the focus of multiple excellent reviews (Haeryfar and Hoskin, 2004; Rege and Hagood, 2006; Herrera-Molina et al., 2013; Leyton and Hagood, 2014; Kumar et al., 2016). In this mini-review, we consider some recent studies that reveal novel aspects of Thy-1 biology that have not been extensively studied, attempt to synthesize these within an emerging view of this molecule, and suggest future areas of exploration.

Thy-1 and INTRACELLULAR SIGNALING

The effects of Thy-1 on cell signaling have been intriguing. An excellent recent review by a pioneer in the field details the signaling effects of Thy-1 relative to its abundance and location on cell surfaces (Morris, 2018). Thy-1 does not function exclusively in a single classic receptor/ligand-type interaction, but additionally can interact with a number of molecules either within the membrane of the same cell (cis) or heterotypically with molecules on the surface of another cell (trans), and in the latter case can affect signaling within the Thy-1-bearing cell or the interacting cell (Herrera-Molina et al., 2013). Known interacting partners include a number of integrin heterodimers, heparin sulfate proteoglycans (e.g., syndecan 4), and some G-protein coupled receptors (Leyton and Hagood, 2014). Interactions of Thy-1 with integrin signaling, particularly though αv heterodimers and Src

family kinase (SFK) activation are perhaps the best characterized, however much remains to be learned. An important challenge has been understanding how Thy-1, as a gycosylphosphatidyl inositol (GPI)-anchored molecule which lacks a transmembrane domain, affects the activation of intracellular signaling molecules such as Src family kinases (SFKs). A recent study carefully dissected the molecular interactions by which Thy-1/αvβ3 interaction regulated SFK activation and cytoskeletal rearrangement in the context of neuron-astrocyte communication (Maldonado et al., 2017). Astrocyte αvβ3 interacts with neuronal Thy-1 in trans to induce neurite retraction. The authors applied two-channel, super-resolution stimulated emission depletion (STED) microscopy combined with single-molecule tracking to show that This interactions slows movement of Thy-1 in the neuronal membrane, promoting formation of aggregates of Thy-1 composed of smaller nanoclusters. These clusters include C-terminal Src kinase (Csk)-binding protein (CBP), a transmembrane scaffolding protein that had previously been shown to confine Thy-1 within lipid raft microdomains (Chen et al., 2009). Cytoplasmic Csk associated with CBP-Thy-1 clusters phosphorylates and inactivates Src, displacing it from these clusters. Interestingly, inactive Src associates with Thy-1 in separate clusters distinct from the Thy-1/CBP/Csk complex. Downstream, inactive Src results in activation of p190RhoGAP, which in turn results in activation of RhoA, resulting in cytoskeletal alterations leading to neurite retraction. A similar pathway downstream of Thy-1/integrin interaction in cis had been previously demonstrated in fibroblasts (Fiore et al., 2015), in which it regulates cell adhesion, cytoskeletal organization, and myofibroblastic differentiation.

Thy-1 interacts with other, non-integrin signaling pathways to modulate cellular phenotype. In fibroblasts, Fas ligand promotes Thy-1/Fas interactions in lipid rafts to promote apoptosis (Liu et al., 2017). This pathway appears important to the resolution of fibrosis, as Thy-$1^{-/-}$ mice fail to resolve fibrosis following bleomycin-induced lung injury, associated with the persistence of apoptosis-resistant myofibroblasts. A study in hepatocellular carcinoma cells showed that the presence of Thy-1, which is a cancer stem cell marker in these tumors, is correlated with enhanced Notch signaling (Luo et al., 2016). This study did not manipulate Thy-1 expression to demonstrate whether this effect is direct or indirect, but this is the first demonstration of an association of Thy-1 with Notch signaling. The broader role of Thy-1 in cancer is complex, as it has been shown to both promote tumorigenesis and to function as a tumor suppressor; this conundrum has been recently reviewed (Kumar et al., 2016). In the context of liver fibrosis Thy-1 was recently found to interact with TGFβRI, indicating a novel mechanism whereby Thy-1 affects TGF-β1 signaling and myofibroblast differentiation (Koyama et al., 2017). Previously, Thy-1 had been shown to inhibit latent TGF-β1 activation in an αv integrin-dependent manner (Zhou et al., 2004, 2010). In liver portal fibroblasts, Thy-1 binding to TGFβRI was shown to be disrupted by mesothelin, indicating two additional novel molecular interactions not previously described for Thy-1 (Koyama et al., 2017). Besides the known integrin- and heparin-binding motifs in the Thy-1 sequence, the mechanisms for many of Thy-1's molecular interactions are not known.

Thy-1 and MECHANOTRANSDUCTION

In addition to responding to chemical signals, most living cells alter their phenotype in response to mechanical stimuli from the external environment. This involves a complex series of molecular interactions from the cell surface to the nucleus (Wang, 2017). Because Thy-1 interacts with numerous integrins and modulates signaling pathways (SFKs, Rho kinases) involved in mechanotransduction, it is well positioned to participate in cellular responses to mechanical cues. Indeed, Thy-1 was found to participate in mechanical signaling in melanoma cells, where it forms a trimolecular complex with α5β1 integrin and syndecan 4 (Fiore et al., 2014). Interestingly, this molecular complex displays binding characteristics of what has been termed "dynamic catch bonds," characterized by rapid bond stiffening and increased binding affinity when force is applied to the bond. More recently, in lung fibroblasts, Thy-1 has been shown to function as a mechanosensor, in that knockdown of Thy-1 results in an inability of fibroblasts to modulate signaling and cell phenotype in response to either increased or decreased matrix stiffness (Fiore et al., 2018). This mechanosensing function is accomplished through Thy-1 interacting with αvβ3 integrin, altering its avidity for ECM binding, while localizing SFKs necessary for downstream mechanosignaling. Loss of Thy-1 in the context of lung injury thus leads to increased integrin activation even in soft provisional ECM, which promotes strain stiffening of matrix, further integrin activation, and progressive fibrosis. *In vivo*, absence of Thy-1 leads to non-resolving fibrosis in a mouse model of lung injury (Fiore et al., 2018). A recent study used molecular force spectroscopy to characterize the mechanical properties of the Thy-1/αvβ3 integrin interaction at the single-molecule level using purified proteins (Burgos-Bravo et al., 2018), and applying mathematical modeling to characterize the slip-bond interaction between these two molecules.

Thy-1 and "STEMNESS"

Thy-1, also known as cluster of differentiation (CD)90, has long been recognized and broadly utilized as a marker of hematopoietic and mesenchymal stem cells. Until recently, however, any mechanistic role in the stem cell phenotype has not been appreciated. Reprogramming of differentiated fibroblasts into induced pluripotent stem cells (iPSCs) using a limited number of transcription factors is one of the major milestones in 21st century biology (Takahashi and Yamanaka, 2006). A study exploring the role of microRNAs (miRs) in fibroblast reprogramming found that after exposure of murine embryonic fibroblasts (MEFs) to the pluripotency-inducing "four factors" (4F), loss of Thy-1 (CD90) was a strong marker of early reprogramming (Li et al., 2014). However, when 4F-infected MEF were sorted into Thy-1^{+} and Thy-1^{-} subpopulations, fully pluripotent iPSCs were highly enriched in the Thy-1^{-}

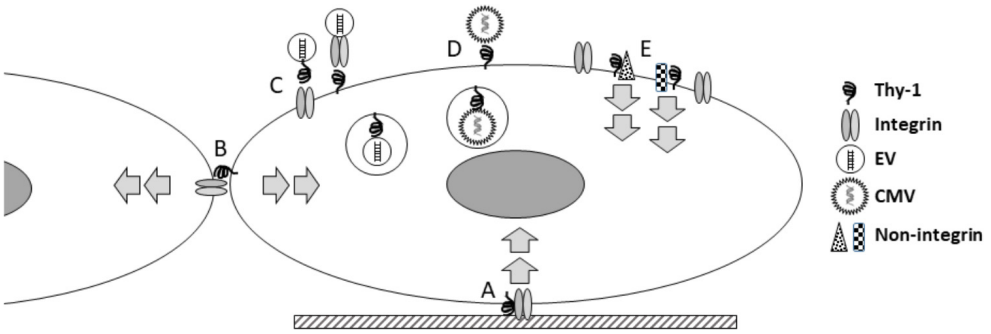

FIGURE 1 | Thy-1 integrates diverse extracellular signals. Thy-1 has been known to participate, via interaction with integrins, in transducing signals from the extracellular matrix **(A)**; more recently this understanding has expanded to include mechanical signals. This type of signaling involves Thy-1 interacting in cis with other molecules within the same cell membrane. Thy-1 is also known to interact in trans with molecules in other cells, in mediating cell-cell interactions **(B)**, with signaling effects in both cells. More recently, Thy-1 has been shown to facilitate binding and internalization of extracellular vesicles (EV; **C**) and cytomegalovirus (CMV; **D**). Increasingly, Thy-1 has been shown to interact with a growing number of non-integrin signaling partners **(E)**. Because the signaling associated with Thy-1 regulates many fundamental cellular processes (stemness, differentiation, migration, and survival), ongoing studies to better understand the molecular mechanisms involved will continue to yield important biological insights about how cells integrate extracellular information.

subpopulation. Loss of Thy-1 was associated with increased expression of miR-135b, which targets a number of ECM-associated genes, including genes involved in TGFβ1 signaling. MEFs which retained Thy-1 expression after 4F infection did not induce miR-135b and retained expression of ECM-associated genes, and had a more limited, multipotent MSC-like phenotype compared to the truly pluripotent Thy-1^{-} iPSCs. This very interesting set of findings suggests that Thy-1 may function as a barrier to pluripotency, and that a mesenchymal Thy-1^{+} phenotype is associated with a microRNA and gene expression profile associated with regulation of ECM-related genes.

Even in MSCs, for which Thy-1 is often used as a marker, its role with regard to differentiation potential is complex. An interesting recent study showed that in human MSCs cultured from numerous sources, lentiviral suppression of Thy-1 increases the capacity for osteoblastic and adipogenic differentiation (Moraes et al., 2016). Complicating matters, MSCs isolated from Thy-$1^{-/-}$ mice were found to have decreased osteoblastic differentiation and increased adipocyte differentiation compared to those from WT mice, consistent with findings of increased adiposity and decreased bone density in Thy-$1^{-/-}$ mice (Picke et al., 2018). These findings suggest that the relationship of Thy-1 expression and mesenchymal differentiation is complex and context-dependent, and that Thy-1 may have many different roles in the pathway from pluripotent stem cells to fully differentiated cells. ECM composition and stiffness are known to affect "stemness," and the roles of Thy-1 as a mechanotransducer and sensor of ECM, as well as a regulator of TGF-β signaling within this context, has yet to be fully elucidated.

Thy-1 and VIRAL ENTRY

The co-evolution of viruses and animal cells has led to a complex array of receptors and mechanisms for viral entry, with strong significance for viral pathogenesis, transplantation, and virus-mediated gene targeting (Baranowski et al., 2003). Many of the viral entry pathways include molecules which regulate cell-cell and cell-matrix interactions, such as integrins. Thus it is not surprising that Thy-1 is involved in this area of biology as well. human cytomegalovirus (HCMV) is a highly prevalent virus that is responsible for human birth defects and organ transplant complications. Thy-1 has recently been shown to play a critical role in entry of HCMV into certain cell types, and more recently this has been demonstrated to involve regulation of clathrin-independent micropinocytosis (Li et al., 2016). Further understanding of this likely primitive mechanism may lead to development of novel inhibitors of viral infection.

Thy-1 and EXTRACELLULAR VESICLES

Membrane-bound vesicles are critical components of intracellular trafficking and signaling. In the past decade there has been tremendous growth in understanding of the roles of secreted extracellular vesicles (EVs), such as exosomes, larger microvesicles, and apoptotic bodies. We had previously demonstrated release of Thy-1 from fibroblasts in response to inflammatory stimuli, and antibody studies indicated that Thy-1 was likely released in a membrane-bound fraction (Hagood et al., 2005; Bradley et al., 2013). Subsequently we demonstrated that MSCs secrete large numbers of EVs, most of which have Thy-1 on the EV surface. These MSC-derived EVs bind to and are internalized by lung fibroblasts, and modulate myofibroblastic differentiation and response to TGFβ1 (Shentu et al., 2017). Antibody-mediated blocking of Thy-1 or β3/β5 integrins prevents EV binding and uptake, as does shRNA-mediated downregulation of Thy-1 and integrins on recipient cells. These findings indicate that Thy-1, either on the EV surface or the recipient cell surface, interacts with integrin on the opposite (cell surface or EV) to facilitate binding, uptake, and delivery of EV content, predominantly miRNA. This is likely similar to the mechanisms for Thy-1-mediated viral entry discussed above. The role of Thy-1 on intracellular vesicles is unknown, but it

has been found in non-caveolar vesicles and neuronal synaptic vesicles (Jeng et al., 1998; Yao et al., 2009).

SUMMARY AND INSIGHTS

How do these seemingly disparate new findings inform our current view of Thy-1? The last half century has produced a substantial body of work on this molecule, during which its roles in a large number of biological fields have become apparent. What insights can be gained from what is known about Thy-1, and what are important questions for future studies? First, Thy-1 is a primitive molecule, conserved to the level of chordates (Mansour et al., 1987), and it is involved in very basic functions (cell-ECM and cell-cell adhesion) important in multicellular biology. The role of Thy-1 in viral entry, EV binding, and signaling all support a broader role for Thy-1 in receiving and processing information from the environment (**Figure 1**). It may be enlightening to consider how Thy-1 might regulate trafficking and processing such information intracellularly. For example, does Thy-1 affect intracellular vesicular functions? Secondly, Thy-1 regulates cell phenotype changes in response to external stimuli, even fundamental characteristics such as pluripotency, differentiation and survival. Understanding the mechanisms by which it does so may yield novel insights into these fundamental processes. Third, the nature of Thy-1's effects seem critically dependent on context, so that what Thy-1 does in one tissue or cell type cannot be simply extrapolated to others. Finally, many but not all of Thy-1's effects involve interaction with integrins, but it is important to remember that Thy-1 also interacts with a growing number of other molecules, and that it may function as part of multi-molecular complexes which affect several important intracellular signaling cascades.

Many unanswered questions remain. Little is known, for example, about how the mode of anchorage of Thy-1 (i.e., its particular GPI anchor) affects its function. Is it merely by regulating its proximity to integrins and other surface molecules, or is the lipid moiety of Thy-1 itself able to interact with other molecules to affect signaling? How important are other post-translational modifications, most notably glycosylation, in some of the seemingly discordant effects of Thy-1 in different contexts? Many of the downstream effects of Thy-1 involve cell phenotype and "identity." These are likely regulated at the level of transcriptional control and epigenetic modifications. Does Thy-1 directly affect nuclear machinery, or are all its effects secondary to its effects on cytoskeletal rearrangements and signaling cascades? With improved understanding of mechanisms comes the ability to target specific aspects of Thy-1 function. However, the translational potential of manipulating Thy-1 remain unexplored. Perhaps the next half century will see important breakthroughs as a result of additional careful study of this enigmatic integrator of extracellular information.

AUTHOR CONTRIBUTIONS

JSH reviewed the relevant literature and wrote the manuscript.

REFERENCES

Baranowski, E., Ruiz-Jarabo, C. M., Pariente, N., Verdaguer, N., and Domingo, E. (2003). Evolution of cell recognition by viruses: a source of biological novelty with medical implications. *Adv. Virus Res.* 62, 19–111.

Bradley, J. E., Chan, J. M., and Hagood, J. S. (2013). Effect of the GPI anchor of human Thy-1 on antibody recognition and function. *Lab. invest.* 93, 365–374. doi: 10.1038/labinvest.2012.178

Bradley, J. E., Ramirez, G., and Hagood, J. S. (2009). Roles and regulation of Thy-1, a context-dependent modulator of cell phenotype. *Biofactors* 35, 258–265. doi: 10.1002/biof.41

Burgos-Bravo, F., Figueroa, N. L., Casanova-Morales, N., Quest, A. F. G., Wilson, C. A. M., and Leyton, L. (2018). Single-molecule measurements of the effect of force on Thy-1/alphavbeta3-integrin interaction using nonpurified proteins. *Mol. Biol. Cell* 29, 326–338. doi: 10.1091/mbc.E17-03-0133

Chen, Y., Veracini, L., Benistant, C., and Jacobson, K. (2009). The transmembrane protein CBP plays a role in transiently anchoring small clusters of Thy-1, a GPI-anchored protein, to the cytoskeleton. *J. Cell Sci.* 122(Pt 21), 3966–3972. doi: 10.1242/jcs.049346

Fiore, V. F., Ju, L., Chen, Y., Zhu, C., and Barker, T. H. (2014). Dynamic catch of a thy-1-alpha5beta1+syndecan-4 trimolecular complex. *Nat. Commun.* 5:4886. doi: 10.1038/ncomms5886

Fiore, V. F., Strane, P. W., Bryksin, A. V., White, E. S., Hagood, J. S., and Barker, T. H. (2015). Conformational coupling of integrin and Thy-1 regulates fyn priming and fibroblast mechanotransduction. *J. Cell Biol.* 211, 173–190. doi: 10.1083/jcb.201505007

Fiore, V. F., Wong, S. S., Tran, C., Tan, C., Xu, W., Sulchek, T., et al. (2018). alphavbeta3 Integrin drives fibroblast contraction and strain stiffening of soft provisional matrix during progressive fibrosis. *JCI Insight* 3:e97597. doi: 10.1172/jci.insight.97597

Haeryfar, S. M., and Hoskin, D. W. (2004). Thy-1: more than a mouse pan-T cell marker. *J. Immunol.* 173, 3581–3588.

Hagood, J. S., Prabhakaran, P., Kumbla, P., Salazar, L., MacEwen, M. W., Barker, T. H., et al. (2005). Loss of fibroblast thy-1 expression correlates with lung fibrogenesis. *Am. J. Pathol.* 167, 365–379.

Herrera-Molina, R., Valdivia, A., Kong, M., Alvarez, A., Cardenas, A., Quest, A. F., et al. (2013). Thy-1-interacting molecules and cellular signaling in cis and trans. *Int. Rev. Cell Mol. Biol.* 305, 163–216. doi: 10.1016/B978-0-12-407695-2.00004-4

Jeng, C. J., McCarroll, S. A., Martin, T. F., Floor, E., Adams, J., Krantz, D., et al. (1998). Thy-1 is a component common to multiple populations of synaptic vesicles. *J. Cell Biol.* 140, 685–698.

Koyama, Y., Wang, P., Liang, S., Iwaisako, K., Liu, X., Xu, J., et al. (2017). Mesothelin/mucin 16 signaling in activated portal fibroblasts regulates cholestatic liver fibrosis. *J. Clin. Invest.* 127, 1254–1270. doi: 10.1172/JCI88845

Kumar, A., Bhanja, A., Bhattacharyya, J., and Jaganathan, B. G. (2016). Multiple roles of CD90 in cancer. *Tumour Biol.* 37, 11611–11622. doi: 10.1007/s13277-016-5112-0

Leyton, L., and Hagood, J. S. (2014). Thy-1 modulates neurological cell-cell and cell-matrix interactions through multiple molecular interactions. *Adv. Neurobiol.* 8, 3–20.

Li, Q., Fischer, E., and Cohen, J. I. (2016). Cell surface thy-1 Contributes to human cytomegalovirus entry via a macropinocytosis-like process. *J. Virol.* 90, 9766–9781. doi: 10.1128/JVI.01092-16

Li, Z., Dang, J., Chang, K. Y., and Rana, T. M. (2014). MicroRNA-mediated regulation of extracellular matrix formation modulates somatic cell reprogramming. *RNA* 20, 1900–1915. doi: 10.1261/rna.043745.113

Liu, X., Wong, S. S., Taype, C. A., Kim, J., Shentu, T. P., Espinoza, C. R., et al. (2017). Thy-1 interaction with Fas in lipid rafts regulates fibroblast apoptosis and lung injury resolution. *Lab. Invest.* 97, 256–267. doi: 10.1038/labinvest.2016.145

Luo, J., Wang, P., Wang, R., Wang, J., Liu, M., Xiong, S., et al. (2016). The notch pathway promotes the cancer stem cell characteristics of CD90+ cells in hepatocellular carcinoma. *Oncotarget* 7, 9525–9537. doi: 10.18632/oncotarget.6672

Maldonado, H., Calderon, C., Burgos-Bravo, F., Kobler, O., Zuschratter, W., Ramirez, O., et al. (2017). Astrocyte-to-neuron communication through integrin-engaged Thy-1/CBP/Csk/Src complex triggers neurite retraction via the RhoA/ROCK pathway. *Biochim. Biophys. Acta Mol. Cell Res.* 1864, 243–254. doi: 10.1016/j.bbamcr.2016.11.006

Mansour, M. H., Negm, H. I., and Cooper, E. L. (1987). Thy-1 evolution. *Dev. Comp. Immunol.* 11, 3–15.

Moraes, D. A., Sibov, T. T., Pavon, L. F., Alvim, P. Q., Bonadio, R. S., Da Silva, J. R., et al. (2016). A reduction in CD90 (THY-1) expression results in increased differentiation of mesenchymal stromal cells. *Stem Cell Res. Ther.* 7:97. doi: 10.1186/s13287-016-0359-3

Morris, R. J. (2018). Thy-1, a pathfinder protein for the post-genomic era. *Front. Cell Dev. Biol.* 6:173. doi: 10.3389/fcell.2018.00173

Picke, A. K., Campbell, G. M., Bluher, M., Krugel, U., Schmidt, F. N., Tsourdi, E., et al. (2018). Thy-1 (CD90) promotes bone formation and protects against obesity. *Sci. Transl. Med.* 10:eaao6806. doi: 10.1126/scitranslmed.aao6806

Rege, T. A., and Hagood, J. S. (2006). Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *Faseb. J.* 20, 1045–1054.

Reif, A. E., and Allen, J. M. (1964). The akr thymic antigen and its distribution in leukemias and nervous tissues. *J. Exp. Med.* 120, 413–433.

Shentu, T. P., Huang, T. S., Cernelc-Kohan, M., Chan, J., Wong, S. S., Espinoza, C. R., et al. (2017). Thy-1 dependent uptake of mesenchymal stem cell-derived extracellular vesicles blocks myofibroblastic differentiation. *Sci. Rep.* 7:18052. doi: 10.1038/s41598-017-18288-9

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Wang, N. (2017). Review of Cellular Mechanotransduction. *J. Phys. D. Appl. Phys.* 50:233002.

Yao, Y., Hong, S., Zhou, H., Yuan, T., Zeng, R., and Liao, K. (2009). The differential protein and lipid compositions of noncaveolar lipid microdomains and caveolae. *Cell Res.* 19, 497–506. doi: 10.1038/cr.2009.27

Zhou, Y., Hagood, J. S., Lu, B., Merryman, W. D., and Murphy-Ullrich, J. E. (2010). Thy-1-integrin alphavbeta5 interactions inhibit lung fibroblast contraction-induced latent TGF-beta1 activation and myofibroblast differentiation. *J. Biol. Chem.* 285, 22382–22393. doi: 10.1074/jbc.M110.126227

Zhou, Y., Hagood, J. S., and Murphy-Ullrich, J. E. (2004). Thy-1 expression regulates the ability of rat lung fibroblasts to activate transforming growth factor-{beta} in response to fibrogenic stimuli. *Am. J. Pathol.* 165, 659–669.

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frontiers in Cell and Developmental Biology

MINI REVIEW
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Thy-1 in Integrin Mediated Mechanotransduction

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The glycosylphosphatidylinositol (GPI) anchored glycoprotein Thy-1 has been prevalently expressed on the surface of various cell types. The biological function of Thy-1 ranges from T cell activation, cell adhesion, neurite growth, differentiation, metastasis and fibrogenesis and has been extensively reviewed elsewhere. However, current discoveries implicate Thy-1 also functions as a key mechanotransduction mediator. In this review, we will be focusing on the role of Thy-1 in translating extracellular mechanic cues into intracellular biological cascades. The mechanotransduction capability of Thy-1 relies on *trans* and *cis* interaction between Thy-1 and RGD-binding integrins; and will be discussed in depth in the review.

Keywords: Thy-1, integrin, *trans* interaction, *cis* interaction, mechanotransduction

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As the smallest member of the immunoglobulin superfamily, Thy-1 (CD90) is a 25–37 kDa glycosyl phosphatidylinositol (GPI) anchored cell membrane protein that bears critical biological functions. The glycoprotein is expressed across many different cell types including fibroblasts, endothelial cells, neuron and hematopoietic cells (Craig et al., 1993; Rege and Hagood, 2006b).Since its discovery decades ago, extensive scrutiny on the glycoprotein has established Thy-1 as an important player in almost every aspect in cellular biology including adhesion, migration, apoptosis, wound healing, tumorigenesis and fibrogenesis (Barker et al., 2004; Sanders et al., 2007, 2008; Barker and Hagood, 2009; Lee et al., 2013). More recently, studies have connected Thy-1 with mechanotransduction, specifically through its interaction with integrins. This mini review will focus on the role of Thy-1 in integrin mediated mechanotransduction, with a broader scope on Thy-1 driven physiological responses via mediating conversion of extracellular biophysical cues into intracellular biochemical signals.

THY-1-INTEGRIN INTERACTION, *CIS* AND *TRANS*

Integrins are a group of adhesion receptors connecting the extracellular matrix (ECM) with the cell cytoskeleton through their bulky, dimeric head domain, a type I transmembrane domain and a relatively small cytoplasmic domain (Luo et al., 2007). Integrins have long been regarded as critical mechanotransducers since the direct engagement between integrins and their ECM ligands is the prerequisite of formation of focal adhesions and cellular contractility. The evidence of Thy-1-integrin interaction began to emerge in the past decade. Potential interactions between Thy-1 and integrin αvβ5 has been proposed as the mechanism of Thy-1 mediated signaling that blocks activation of TGF-β (Herrera-Molina et al., 2013). Similarly, Thy-1 positive lung fibroblasts are resistant to TGF-β activation induced lung fibrosis, implicating possible role of Thy-1 in suppressing αvβ6 mediated TGF-β activation (Zhou et al., 2004). Direct interactions have been shown between Thy-1 and integrin αvβ3 on astrocytes (Leyton et al., 2001). The interaction is mediated through the RLD motif on the recombinant Thy-1-FC molecule and the engagement

between Thy-1 and αvβ3 can promote focal adhesion formation as well as FAK phosphorylation. Another study later discovered that this *trans* interaction between Thy-1 and integrin αvβ3 induces Thy-1 microclustering and colocalization with Csk-binding protein (CBP) while displacing Src kinase from these clusters at the same time (Maldonado et al., 2017). Melanoma cells have also been seen to exploit Thy-1 expressed by vascular endothelial cells for adhesion and subsequent tumor metastasis, presumably through Thy-1- αvβ3 interaction (Schubert et al., 2013). αvβ3 is not the only integrin that has shown capability to interact with Thy-1. Thy-1-α5β1 and syndecan4 can form triplex and behave as a catch bond (Fiore et al., 2014).

While *trans* interactions between Thy-1 and integrin apparently mediates mechanotransduction, little is known regarding the impact of *cis* interaction until lately. In a study published by Fiore and his colleagues, Thy-1 is found to interact with integrin αvβ3 in *cis* on the surface of lung fibroblasts (Fiore et al., 2015). The interaction helps to keep the integrin in a low affinity, bent conformation. Moreover, the interaction facilitates Fyn, a member of SFK critical in mechanosignaling, recruitment to focal adhesions while also keeps c-Src activity under check through recruitment of CBP.

TRANS INTERACTION BETWEEN THY-1 AND INTEGRIN αvβ3 MEDIATES MECHANOTRANSDUCTION

Thy-1 has been shown to support cell adhesion through *trans* interaction with integrin. Immobilized Thy-1 is capable to function as ligand for integrin αvβ3 and support cell adhesion in a Mn^{2+} dependent manner. On the cell membrane, interactions between αvβ3 on DITNC1 astrocytes and Thy-1 on neuron cells support cell adhesion but inhibit neuron cell differentiation and neurite extension (Herrera-Molina et al., 2012). Immobilized recombinant αvβ3-FC functions similarly and induces clustering of Thy-1 on neuron cell surface. It has been proposed that such a *trans* interaction triggered redistribution/clustering of Thy-1 leads to inactivation of Src through Thy-1 mediated CBP recruitment. Thy-1 mediated cell-cell interaction has also been found to be critical for melanoma cell adhesion and metastasis. Thy-1 deficient mice showed significantly reduced metastasis sites due to ablation of Thy-1 mediated melanoma cell adhesion on Endothelial cells (Schubert et al., 2013). When mediating cell-cell adhesion, Thy-1 not only needs to interact with integrin αvβ3, but also need to bring in Syndecan4, a lipid raft protein that binds to a heparin-binding domain on Thy-1. The interaction between Thy-1 and Syndecan4 itself is not sufficient to induce Rac-1 RhoGTPase activation; however, the binding is required for the Thy-1- αvβ3 interaction to support cell adhesion and migration (Kong et al., 2013). It's worth noting that the Thy-1- αvβ3 interaction alone indeed triggers phosphorylation of Akt, indicating that cell-cell *trans* interaction through Thy-1 and integrin could promote cell viability/survival but is not sufficient to generate mechano-signal transduction. Interestingly, while surface Thy-1 clustering induced by integrin αvβ3 generates inhibitory signal in neuron cells, Thy-1 crosslinking by mAb induces Ca^{2+} influx and proliferation in T lymphocytes (Kroczek et al., 1986; Conrad et al., 2009). The seemingly paradoxical evidence implicates highly context dependent nature of Thy-1 function.

Thy-1-FC conjugated beads are sufficient to induce enhanced formation of focal adhesions and elevated tyrosine phosphorylation of $p130^{cas}$ and FAK (Leyton et al., 2001). A Thy-1-CBP-RhoA-ROCK axis has been proposed to induce astrocyte retraction and RhoA dependent actin stress fiber formation (Avalos et al., 2004; Maldonado et al., 2017). The phenomenon is induced via Thy-1-FC conjugated protein A beads, implicating that clustered Thy-1 is likely required to mediate the *trans*-interaction based mechanotransduction through integrin αvβ3. Unlike other traditional integrin ligands, Thy-1 is a monovalent molecule and its RLD motif likely binds with integrin at lower affinity. Therefore, clustering of Thy-1 and presence of potential binding partners in addition to integrin (e.g., syndecan4) could be essential for Thy-1 mediated cell-cell interaction and mechanotransduction. This is particularly plausible considering that immobilized Thy-1-FC can't support cell adhesion without the presence of Mn^{2+} whereas conjugated (and thus "clustered") Thy-1-FC beads successfully induced focal adhesion assembly in a RhoA-ROCK dependent pathway (Leyton et al., 2001; Avalos et al., 2004). Interaction with ECM ligands induces integrin clustering which is the key event in cell adhesion and migration. It is known that integrin clustering is dependent on $PI(4,5,)P_2$ and Talin (Cluzel et al., 2005; Saltel et al., 2009) while syndecan4 helps retention of $PI(4,5,)P_2$ in cell membrane (Kwon et al., 2009). Therefore, Thy-1, integrin αvβ3 and syndecan4 work synergistically to mediate mechanotransduction through cell-cell interaction. Further downstream, this trimolecular complex also regulates RhoA GTPase mainly through modulating p^{190}GAP phosphorylation and distribution. Syndecan4 and integrin α5β1 have been shown to regulate p^{190}GAP membrane distribution and Src-dependent tyrosine phosphorylation, respectively (Bass et al., 2008). The coordinated interaction subsequently leads to suppressed RhoA activity and cell migration. However, introduction of Thy-1 causes a reduction of Src activity and downregulation of p^{190}GAP, which leads to higher RhoA activity, stable adhesion and enhanced stress fiber formation (Barker et al., 2004). The phenomenon can be attributed, in part, to the recruitment of CBP by Thy-1 to integrin membrane proximity, which leads to inhibitory phosphorylation of Src kinase by CBP interacting Csk. More interestingly, the Thy-1- α5β1-Syndecan4 trimolecular complex not only delivers mechano-related biochemical signaling coordinately but also physically interprets force directly (Fiore et al., 2014). When Thy-1 binds to either α5β1 integrin or Syndecan4 alone, both interactions behave as classic slip bond, meaning that the lifetime of the interactions decreases with force application. However, the trimolecular bond expresses a unique catch bond feature–described as "dynamic catch" by the authors. The mechanism behind the phenomenal has been proposed as a sudden bond stiffening from an acquired contribution of the syndecan4-Thy-1 interaction, once the force load reaches a ∼15 pN threshold. Before reaching the threshold, α5β1-Thy-1 interaction bears the majority of the force whereas after the threshold, due to force-induced extension of the GAG

motif on Syndecan4, both α5β1 and Syndecan4 start to resist force at full load. Taken together, the Thy-1-α5β1-Syndecan4 complex mediates mechanotransduction both at the single molecule biophysical level and at the cell biochemical level.

MECHANOTRANSDUCTION IN *CIS*

In contrast to only the induction of focal adhesions and promotion of FAK activation seen in *trans*, *cis* interaction between Thy-1 and integrin is more complicated, providing both a tonic inhibition, but also facilitating efficient mechanosignaling in the focal adhesion (Fiore et al., 2015). The Thy-1-αvβ3 interaction shifts the dynamic equilibrium of integrin conformation toward a bent-closed state. This effectively reduces integrin avidity for its extracellular ligand; Thy-1 is a weak inhibitor of integrin in *cis*. Remarkably, the Thy-1-αvβ3 interaction physically couples unbound integrin to lipid raft microdomains containing critical signaling molecules. Thus, Thy-1 facilitates co-clustering of lipid raft proteins with focal adhesions enabling proper mechanosensing in fibroblasts. Integrin mediated mechanotransduction relies on ECM ligand engagement and subsequent integrin clustering, which leads to self-activation of FAK and Src, resulting in downstream RhoA activation and cellular contractility (Hu and Luo, 2013). More specifically, by keeping integrin in the bend-low affinity conformation, Thy-1 not only constraints the ligand accessibility for integrin but also limits the likelihood of ECM ligand binding independent self-clustering and thus reduces the overall integrin avidity. Thy-1 keeps c-Src activity in check through recruitment of the lipid raft protein CBP, which leads to recruitment of Src inhibitor Csk; concurrently the lipid raft-associated Src-family member Fyn is brought to the focal adhesion enabling a prompt mechanosignaling response after ligand engagement (Fiore et al., 2015). It is worth noting that this Thy-1-mediated mechanosensing requires proper lipid raft location, as replacing the GPI anchor with a CD8 transmembrane domain greatly reduced the ability of cells to appropriately respond to environmental rigidity. Lipid rafts have been widely regarded as a critical participant in mechanotransduction (Head et al., 2014). Colocalization of Fyn, CBP and another Thy-1 interacting protein Reggie1/2 on non-caveolar lipid raft has been reported previously (Stuermer et al., 2001; Deininger et al., 2003). Moreover, Fyn has been shown to be able to both interact with FAK in early integrin mediated adhesion and phosphorylate CBP, resulting in subsequent recruitment/activation of Csk (Yasuda et al., 2002; Maksumova et al., 2005; Baillat et al., 2008). Adding the evidence together, Thy-1 likely functions as a lipid raft coupler, recruiting Fyn and CBP to the focal adhesion to regulate basal Src activity through Csk. In addition to promote RhoA activity through downregulating c-Src dependent p^{190}GAP activity, Fyn has also shown to directly phosphorylate and activate Rho guanine nucleotide exchange factor (GEF) in response to integrin mediated force transduction, resulting in a more direct activation of RhoA (Guilluy et al., 2011). Importantly, the activity of Rho GTPase is required for ECM stiffness induced nucleus translocation of Yap/Taz, which drives mechano-activation of fibroblast and fibrosis (Dupont et al., 2011; Liu et al., 2015). Taking together, the direct and indirect regulatory role of Fyn over RhoA activity makes it a core modulator of force-induced cellular response.

The third way Thy-1 appears to impact mechanotransduction is through regulating the TGF-β pathway. TGF-β-SMAD2/3/4 is well established as the main signaling route to induce mechano-related cellular responses including proliferation, cellular contraction and ECM deposition. The signaling axis is also the main driving force in fibrosis. It has been reported that Thy-1 null c57BL/6 mice were more prone to develop severe lung fibrosis after bleomycin treatment (Hagood et al., 2005). Thy-1 negative fibroblasts are more responsive toward inflammatory cytokines like TGF-β whereas Thy-1 positive cells are resistant to similar treatments. The difference does not appear to be due to downstream signal transduction of TGF-β but instead to higher latent TGF-β activation in Thy-1 negative cells (Zhou et al., 2004), potentially through Thy-1 stabilization of integrin's bent conformation as described above. Likewise, induction of MMP9 by TGF-β has been observed in Thy-1 negative fibroblasts but not in Thy-1 positive fibroblasts, implicating Thy-1 as an important suppressor in MMP9 induced latent TGF-β activation– the positive feedback loop that efficiently enhances TGF-β signaling (Ramirez et al., 2011). The interaction between Thy-1 and integrin αvβ5 has been proposed as a mechanism to constrain latent TGF-β activation by the integrin (Zhou et al., 2010). The study, however, failed to reveal if the inhibition is caused by *cis* interaction between the two molecules or *trans*. It is conceivable that by keeping TGF-β activating integrins (αvβ5 and αvβ6) in a low affinity conformation, Thy-1 can reduce activation of endogenous TGF-β, enabling a cellular "brake" to TGF-β.

CONCLUSION

Thy-1 bears a vast range of functionality, affecting T cell activation, proliferation, differentiation, neuron regeneration, adhesion and fibrosis (Rege and Hagood, 2006a,b). Interestingly, many of these functions are overlapping with integrin functionalities such as immunological synapse formation (αLβ2; Springer and Dustin, 2012), proliferation, adhesion, etc. The dual integrin interacting pattern (*trans* and *cis*) makes Thy-1 a key mechanoregulator through its integrin interaction capacity. The subsequent biological impacts of Thy-1-integrin interactions can be further categorized as either on the plasmamembrane or in the cytosol.

On the plasma membrane, Thy-1 exerts profound impact in a direct manner. On the surface of neurons, Thy-1 directly binds to astrocyte integrin αvβ3 in *trans*. The interaction triggers Thy-1 clustering and suppresses neuron outgrowth (Leyton et al., 2001). Thy-1 dependent cell adhesion and migration is also mediated through the *trans* interaction between Thy-1 and integrin, namely αvβ3, αXβ2, and αMβ2 (Rege and Hagood, 2006a). Therefore, the *trans* interaction mediates mechanotransduction in the context of cell-cell interaction, which result in either clustering of Thy-1 and subsequent suppression of c-Src (**Figure 1**①) or directly force

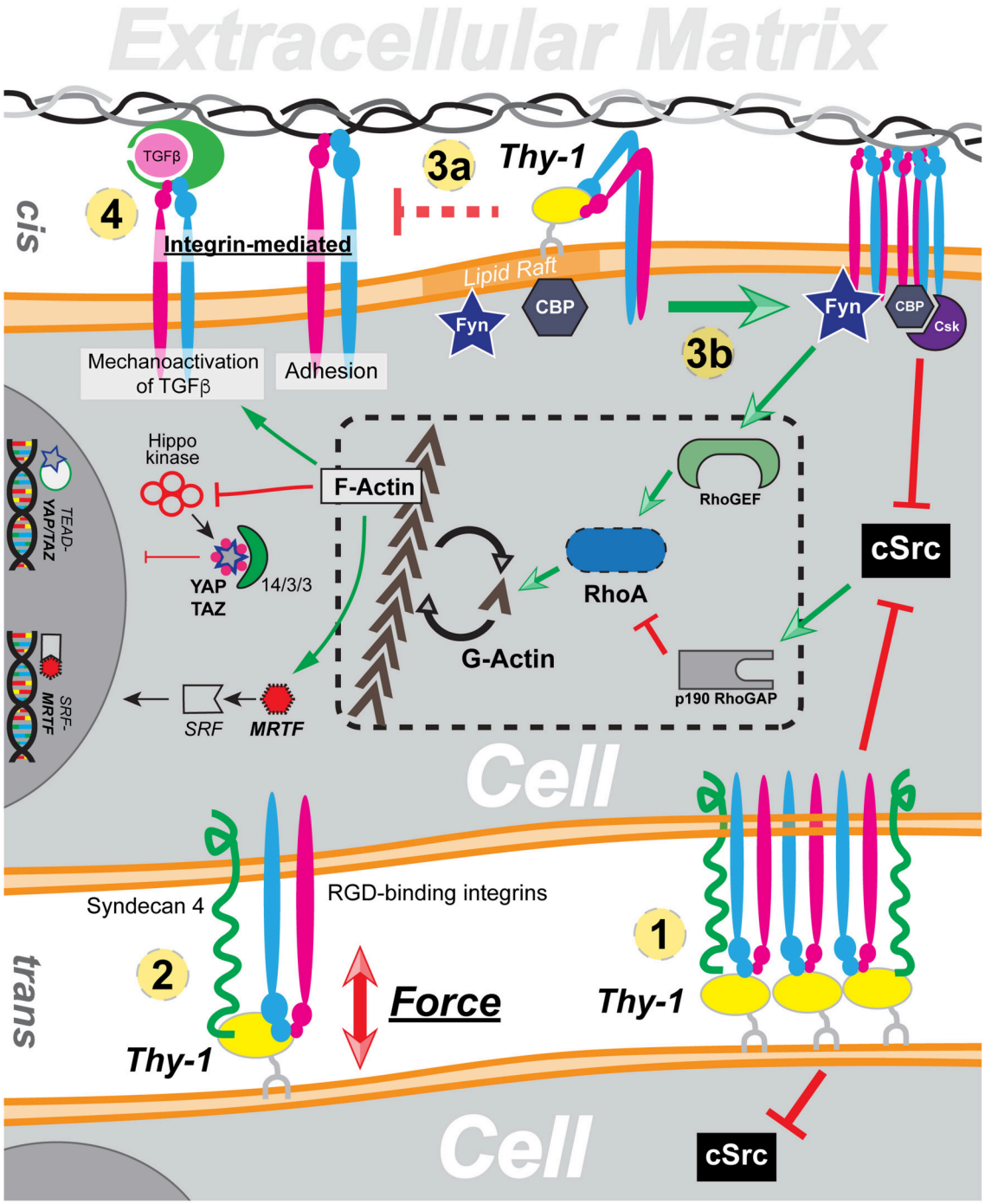

FIGURE 1 | A global overview of Thy-1's functionality in mechanotransduction through cis and trans interaction with integrin. ① Trans interaction with integrin induces Thy-1 clustering and Src inhibition. ② Thy-1-integrin-Syndecan4 triplex directly responds to force by forming a dynamic catch bond. ③ Direct cis interaction between Thy-1 and integrin results in reduction of integrin affinity (a) and downstream regulation of integrin mediated mechanotransduction (b). ④ Thy-1-integrin cis interaction also suppresses integrin-dependent TGF-β activation.

transduction (**Figure 1**②). The *cis* interaction, on the other hand, plays a more inhibitive/regulatory role in integrin mediated mechanotransduction. Through direct binding to integrin through its RLD motif, Thy-1 can restrict integrin by promoting its bent conformation, due to the proximity of the RLD motif against the plasma membrane. Furthermore, through interaction with integrin, Thy-1 can also effectively reduce overall integrin avidity toward ECM ligands (**Figure 1**③a)

and at the same time, inhibit integrin-mediated latent TGF-β activation (**Figure 1**④).

The impacts of Thy-1 on cytoplasmic mechanotransduction pathway, on the other hand, are indirect due to the lack of a Thy-1 cytoplasmic domain. In addition to regulating integrin affinity/avidity through direct *cis*-interaction, Thy-1-integrin binding also facilitates phosphorylation and recruitment of CBP through Fyn, a lipid raft Src family kinase (SFK) recruited to the focal adhesion by Thy-1. Subsequently, Csk is recruited by CBP, leading to phosphorylate the c-terminus of and inactivate c-Src. The c-Src inhibition subsequently leads to reduced p^{190}GAP activity and elevated RhoA-dependent actin stress fiber assembly. It has been widely described that RhoA/ROCK controlled cellular G-actin pool dynamics directly regulates nuclear translocation and activation of MRTF (Miralles et al., 2003; Fan et al., 2007; Vartiainen et al., 2007). Therefore, Thy-1 mediated downregulation of Src activity can result in nuclear accumulation of active MRTF in response to extracellular tension by reducing availability of MRTF-inhibitory G-actin (**Figure 1**③b). Similarly, both Hippo dependent and independent Yap/Taz signal transduction are also tightly regulated by RhoA mediated F-actin stress fiber assembly (Dupont et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011). Besides a relatively "slower" pathway to regulate RhoA activity through Src, Fyn has also been shown to directly activate Rho GEF LARG and thus enables swift early cellular response toward extracellular mechanic cues through RhoA. In sum, through indirectly manipulating RhoA activity and subsequent equilibrium between G-actin and F-actin, Thy-1 functions as a key regulator of cellular mechanotransduction.

The impact of Thy-1 on mechanotransduction is likely the fundamental mechanism behind its broad functionality. This mechano-based regulatory mechanism not only affects cellular behavior but also profoundly influence tissue development and cell differentiation. Thy-1 negative fibroblasts are more sensitive to inflammatory cytokines and more likely to differentiate into myofibroblasts (Sanders et al., 2007). Thy-1 deficiency also leads to poor osteogenesis in mouse due to altered Wnt pathway (Picke et al., 2018). Without Thy-1, mouse mesenchymal stem cells (MSC) are more likely to differentiate into adipocytes instead of osteoblasts (Picke et al., 2018). These discoveries strongly suggest that Thy-1, through integrin mediated mechanotransduction, significantly influences differentiation and cell fate determination. Recently it has been reported that integrin αvβ3 signaling potentiates fibrotic activation of lung fibroblast (Fiore et al., 2018). In the study, Thy-1 KD induced fibroblast stiffening and promoted MRTF nucleus translocation with enhanced cellular contractility. Elevated αvβ3 staining was observed in both Thy-1 KD cells and in Thy-1 null mice treated with bleomycin to induce lung fibrosis, implicating strong correlation between lung fibrogenesis, Thy-1 loss and dysregulated integrin αvβ3 signaling.

Unlike other mechanotransducing molecules, Thy-1 is capable of mediating mechanotransduction through *trans* AND *cis* interactions with integrins, making the GPI anchored protein a unique mechano mediator. The Thy-1 mediated mechanotransduction is highly context dependent. The *trans* molecular coupling of Thy-1 with integrin and Syndecan4 is necessary to generate full strength of force as well as cellular contractile formation. Meanwhile, the lipid raft GPI anchor is an absolute requirement for Thy-1 *cis* mechanotransduction, emphasizing the importance of lipid environment for proper Thy-1 functionality. Considering that the function of Thy-1 is also highly cell type dependent, it is conceivable that differential membrane protein coupling and subtle change in lipid raft composition could serve as a fine-tuned regulatory mechanism of Thy-1 mediated mechanotransduction. Therefore, Thy-1 could potentially be coupling with other not-yet-identified lipid raft proteins directly or indirectly and thus regulating a wide range of mechano-related cellular response spanning from ECM remodeling to cell differentiation and determination. More studies are needed to fully understand the role of Thy-1 in the context of mechanotransduction.

AUTHOR CONTRIBUTIONS

PH and TB conceived the idea and wrote the manuscript. TB conceptualized and created the figure.

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REFERENCES

Avalos, A. M., Arthur, W. T., Schneider, P., Quest, A. F., Burridge, K., and Leyton, L. (2004). Aggregation of integrins and RhoA activation are required for Thy-1-induced morphological changes in astrocytes. *J. Biol. Chem.* 279, 39139–39145. doi: 10.1074/jbc.M403439200

Baillat, G., Siret, C., Delamarre, E., and Luis, J. (2008). Early adhesion induces interaction of FAK and Fyn in lipid domains and activates raft-dependent Akt signaling in SW480 colon cancer cells. *Biochim. Biophys. Acta* 1783, 2323–2331. doi: 10.1016/j.bbamcr.2008.08.008

Barker, T. H., Grenett, H. E., MacEwen, M. W., Tilden, S. G., Fuller, G. M., Settleman, J., et al. (2004). Thy-1 regulates fibroblast focal adhesions, cytoskeletal organization and migration through modulation of p190 RhoGAP and Rho GTPase activity. *Exp. Cell Res.* 295, 488–496. doi: 10.1016/j.yexcr.2004.01.026

Barker, T. H., and Hagood, J. S. (2009). Getting a grip on Thy-1 signaling. *Biochim. Biophys. Acta* 1793, 921–923. doi: 10.1016/j.bbamcr.2008.10.004

Bass, M. D., Morgan, M. R., Roach, K. A., Settleman, J., Goryachev, A. B., and Humphries, M. J. (2008). p190RhoGAP is the convergence point of adhesion signals from alpha 5 beta 1 integrin and syndecan-4. *J. Cell Biol.* 181, 1013–1026. doi: 10.1083/jcb.200711129

Cluzel, C., Saltel, F., Lussi, J., Paulhe, F., Imhof, B. A., and Wehrle-Haller, B. (2005). The mechanisms and dynamics of αvβ3 integrin clustering in living cells. *J. Cell Biol.* 171, 383–392. doi: 10.1083/jcb.200503017

Conrad, D. M., Furlong, S. J., Doucette, C. D., Boudreau, R. T., and Hoskin, D. W. (2009). Role of mitogen-activated protein kinases in

Thy-1-induced T-lymphocyte activation. *Cell. Signal.* 21, 1298–1307. doi: 10.1016/j.cellsig.2009.03.014

Craig, W., Kay, R., Cutler, R. L., and Lansdorp, P. M. (1993). Expression of Thy-1 on human hematopoietic progenitor cells. *J. Exp. Med.* 177, 1331–1342. doi: 10.1084/jem.177.5.1331

Deininger, S. O., Rajendran, L., Lottspeich, F., Przybylski, M., Illges, H., Stuermer, C. A., et al. (2003). Identification of teleost Thy-1 and association with the microdomain/lipid raft reggie proteins in regenerating CNS axons. *Mol. Cell. Neurosci.* 22, 544–554. doi: 10.1016/S1044-7431(03)00028-9

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., et al. (2011). Role of YAP/TAZ in mechanotransduction. *Nature* 474, 179–183. doi: 10.1038/nature10137

Fan, L., Sebe, A., Peterfi, Z., Masszi, A., Thirone, A. C., Rotstein, O. D., et al. (2007). Cell contact-dependent regulation of epithelial-myofibroblast transition via the rho-rho kinase-phospho-myosin pathway. *Mol. Biol. Cell* 18, 1083–1097. doi: 10.1091/mbc.e06-07-0602

Fiore, V. F., Ju, L., Chen, Y., Zhu, C., and Barker, T. H. (2014). Dynamic catch of a Thy-1-α5bβ1+syndecan-4 trimolecular complex. *Nat. Commun.* 5, 4886. doi: 10.1038/ncomms5886

Fiore, V. F., Strane, P. W., Bryksin, A. V., White, E. S., Hagood, J. S., and Barker, T. H. (2015). Conformational coupling of integrin and Thy-1 regulates Fyn priming and fibroblast mechanotransduction. *J. Cell Biol.* 211, 173–190. doi: 10.1083/jcb.201505007

Fiore, V. F., Wong, S. S., Tran, C., Tan, C., Xu, W., Sulchek, T., et al. (2018). αvβ3 Integrin drives fibroblast contraction and strain stiffening of soft provisional matrix during progressive fibrosis. *JCI Insight* 3:97597. doi: 10.1172/jci.insight.97597

Guilluy, C., Swaminathan, V., Garcia-Mata, R., O'Brien, E. T., Superfine, R., and Burridge, K. (2011). The Rho GEFs LARG and GEF-H1 regulate the mechanical response to force on integrins. *Nat. Cell Biol.* 13, 722–727. doi: 10.1038/ncb2254

Hagood, J. S., Prabhakaran, P., Kumbla, P., Salazar, L., MacEwen, M. W., Barker, T. H., et al. (2005). Loss of fibroblast Thy-1 expression correlates with lung fibrogenesis. *Am. J. Pathol.* 167, 365–379. doi: 10.1016/S0002-9440(10)62982-3

Head, B. P., Patel, H. H., and Insel, P. A. (2014). Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling. *Biochim. Biophys. Acta* 1838, 532–545. doi: 10.1016/j.bbamem.2013.07.018

Herrera-Molina, R., Frischknecht, R., Maldonado, H., Seidenbecher, C. I., Gundelfinger, E. D., Hetz, C., et al. (2012). Astrocytic αVβ3 integrin inhibits neurite outgrowth and promotes retraction of neuronal processes by clustering Thy-1. *PLoS ONE* 7:e34295. doi: 10.1371/journal.pone.0034295

Herrera-Molina, R., Valdivia, A., Kong, M., Alvarez, A., Cardenas, A., Quest, A. F., et al. (2013). Thy-1-interacting molecules and cellular signaling in cis and trans. *Int. Rev. Cell Mol. Biol.* 305, 163–216. doi: 10.1016/B978-0-12-407695-2.00004-4

Hu, P., and Luo, B. H. (2013). Integrin bi-directional signaling across the plasma membrane. *J. Cell. Physiol.* 228, 306–312. doi: 10.1002/jcp.24154

Kong, M., Munoz, N., Valdivia, A., Alvarez, A., Herrera-Molina, R., Cardenas, A., et al. (2013). Thy-1-mediated cell-cell contact induces astrocyte migration through the engagement of αVβ3 integrin and syndecan-4. *Biochim. Biophys. Acta* 1833, 1409–1420. doi: 10.1016/j.bbamcr.2013.02.013

Kroczek, R. A., Gunter, K. C., Germain, R. N., and Shevach, E. M. (1986). Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. *Nature* 322, 181–184. doi: 10.1038/322181a0

Kwon, S., Son, H., Choi, Y., Lee, J. H., Choi, S., Lim, Y., et al. (2009). Syndecan-4 promotes the retention of phosphatidylinositol 4,5-bisphosphate in the plasma membrane. *FEBS Lett.* 583, 2395–2400. doi: 10.1016/j.febslet.2009.06.039

Lee, M. J., Shin, J. O., and Jung, H. S. (2013). Thy-1 knockdown retards wound repair in mouse skin. *J. Dermatol. Sci.* 69, 95–104. doi: 10.1016/j.jdermsci.2012.11.009

Leyton, L., Schneider, P., Labra, C. V., Ruegg, C., Hetz, C. A., Quest, A. F., et al. (2001). Thy-1 binds to integrin β3 on astrocytes and triggers formation of focal contact sites. *Curr. Biol.* 11, 1028–1038. doi: 10.1016/S0960-9822(01)00262-7

Liu, F., Lagares, D., Choi, K. M., Stopfer, L., Marinkovic, A., Vrbanac, V., et al. (2015). Mechanosignaling through YAP and TAZ drives fibroblast activation and fibrosis. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 308, L344–357. doi: 10.1152/ajplung.00300.2014

Luo, B. H., Carman, C. V., and Springer, T. A. (2007). Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* 25, 619–647. doi: 10.1146/annurev.immunol.25.022106.141618

Maksumova, L., Le, H. T., Muratkhodjaev, F., Davidson, D., Veillette, A., and Pallen, C. J. (2005). Protein tyrosine phosphatase α regulates Fyn activity and Cbp/PAG phosphorylation in thymocyte lipid rafts. *J. Immunol.* 175, 7947–7956. doi: 10.4049/jimmunol.175.12.7947

Maldonado, H., Calderon, C., Burgos-Bravo, F., Kobler, O., Zuschratter, W., Ramirez, O., et al. (2017). Astrocyte-to-neuron communication through integrin-engaged Thy-1/CBP/Csk/Src complex triggers neurite retraction via the RhoA/ROCK pathway. *Biochim Biophys Acta Mol Cell Res* 1864, 243–254. doi: 10.1016/j.bbamcr.2016.11.006

Miralles, F., Posern, G., Zaromytidou, A. I., and Treisman, R. (2003). Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* 113, 329–342. doi: 10.1016/S0092-8674(03)00278-2

Picke, A. K., Campbell, G. M., Bluher, M., Krugel, U., Schmidt, F. N., Tsourdi, E., et al. (2018). Thy-1 (CD90) promotes bone formation and protects against obesity. *Sci. Transl. Med.* 10:eaao6806. doi: 10.1126/scitranslmed.aao6806

Ramirez, G., Hagood, J. S., Sanders, Y., Ramirez, R., Becerril, C., Segura, L., et al. (2011). Absence of Thy-1 results in TGF-β induced MMP-9 expression and confers a profibrotic phenotype to human lung fibroblasts. *Lab. Invest.* 91, 1206–1218. doi: 10.1038/labinvest.2011.80

Rege, T. A., and Hagood, J. S. (2006a). Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *FASEB J.* 20, 1045–1054. doi: 10.1096/fj.05-5460rev

Rege, T. A., and Hagood, J. S. (2006b). Thy-1, a versatile modulator of signaling affecting cellular adhesion, proliferation, survival, and cytokine/growth factor responses. *Biochim. Biophys. Acta* 1763, 991–999. doi: 10.1016/j.bbamcr.2006.08.008

Saltel, F., Mortier, E., Hytonen, V. P., Jacquier, M. C., Zimmermann, P., Vogel, V., et al. (2009). New PI(4,5)P2- and membrane proximal integrin-binding motifs in the talin head control β3-integrin clustering. *J. Cell Biol.* 187, 715–731. doi: 10.1083/jcb.200908134

Sanders, Y. Y., Kumbla, P., and Hagood, J. S. (2007). Enhanced myofibroblastic differentiation and survival in Thy-1(-) lung fibroblasts. *Am. J. Respir. Cell Mol. Biol.* 36, 226–235. doi: 10.1165/rcmb.2006-0178OC

Sanders, Y. Y., Pardo, A., Selman, M., Nuovo, G. J., Tollefsbol, T. O., Siegal, G. P., et al. (2008). Thy-1 promoter hypermethylation: a novel epigenetic pathogenic mechanism in pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* 39, 610–618. doi: 10.1165/rcmb.2007-0322OC

Sansores-Garcia, L., Bossuyt, W., Wada, K., Yonemura, S., Tao, C., Sasaki, H., et al. (2011). Modulating F-actin organization induces organ growth by affecting the Hippo pathway. *EMBO J.* 30, 2325–2335. doi: 10.1038/emboj.2011.157

Schubert, K., Gutknecht, D., Koberle, M., Anderegg, U., and Saalbach, A. (2013). Melanoma cells use Thy-1 (CD90) on endothelial cells for metastasis formation. *Am. J. Pathol.* 182, 266–276. doi: 10.1016/j.ajpath.2012.10.003

Springer, T. A., and Dustin, M. L. (2012). Integrin inside-out signaling and the immunological synapse. *Curr. Opin. Cell Biol.* 24, 107–115. doi: 10.1016/j.ceb.2011.10.004

Stuermer, C. A., Lang, D. M., Kirsch, F., Wiechers, M., Deininger, S. O., and Plattner, H. (2001). Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and−2. *Mol. Biol. Cell* 12, 3031–3045. doi: 10.1091/mbc.12.10.3031

Vartiainen, M. K., Guettler, S., Larijani, B., and Treisman, R. (2007). Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL. *Science* 316, 1749–1752. doi: 10.1126/science.1141084

Wada, K., Itoga, K., Okano, T., Yonemura, S., and Sasaki, H. (2011). Hippo pathway regulation by cell morphology and stress fibers. *Development* 138, 3907–3914. doi: 10.1242/dev.070987

Yasuda, K., Nagafuku, M., Shima, T., Okada, M., Yagi, T., Yamada, T., et al. (2002). Cutting edge: fyn is essential for tyrosine phosphorylation of Csk-binding protein/phosphoprotein associated with glycolipid-enriched

microdomains in lipid rafts in resting T cells. *J. Immunol.* 169, 2813–2817. doi: 10.4049/jimmunol.169.6.2813

Zhou, Y., Hagood, J. S., Lu, B., Merryman, W. D., and Murphy-Ullrich, J. E. (2010). Thy-1-integrin αvβ5 interactions inhibit lung fibroblast contraction-induced latent transforming growth factor-β1 activation and myofibroblast differentiation. *J. Biol. Chem.* 285, 22382–22393. doi: 10.1074/jbc.M110.126227

Zhou, Y., Hagood, J. S., and Murphy-Ullrich, J. E. (2004). Thy-1 expression regulates the ability of rat lung fibroblasts to activate transforming growth factor-beta in response to fibrogenic stimuli. *Am. J. Pathol.* 165, 659–669. doi: 10.1016/S0002-9440(10)63330-5

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REVIEW
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CD90/Thy-1, a Cancer-Associated Cell Surface Signaling Molecule

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CD90 is a membrane GPI-anchored protein with one Ig V-type superfamily domain that was initially described in mouse T cells. Besides the specific expression pattern and functions of CD90 that were described in normal tissues, i.e., neurons, fibroblasts and T cells, increasing evidences are currently highlighting the possible involvement of CD90 in cancer. This review first provides a brief overview on CD90 gene, mRNA and protein features and then describes the established links between CD90 and cancer. Finally, we report newly uncovered functional connections between CD90 and endoplasmic reticulum (ER) stress signaling and discuss their potential impact on cancer development.

Keywords: THY-1, CD90, cancer, invasion, migration, ER stress, IRE1

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INTRODUCTION

Thy-1/CD90 was first identified in 1964 on mouse T lymphocytes (Reif and Allen, 1964a,b) and then on rat thymocytes and neural cells (Barclay et al., 1976). Since then, more than 10, 000 publications refer to CD90 mainly in rodent and human species (**Figure 1A**). The *CD90* gene is conserved from fish to mammal (vertebrates; **Figure 1B**), and homologs have been even described in some invertebrates such as squids, tunicates, and worms (Cooper and Mansour, 1989). *CD90* gene organization including promoter region and methylation sites was further described and reviewed in Barclay et al. (1976); Seki et al. (1985); Cooper and Mansour (1989). Importantly, the *CD90* promoter is often considered to be specifically activated in the brain. Consequently, the *CD90* promoter has routinely been used to drive "brain specific" expression of proteins in mice (Feng et al., 2000). The mouse and human CD90 protein are highly similar sharing 66% identity (**Figure 1C**).

The CD90 protein is a small membrane glycophosphatidylinositol (GPI) anchored protein of 25 to 37 kDa, heavily N-glycosylated on two or three sites in human and mouse, respectively. One third of the CD90 molecular mass is linked to its glycosylation level (Pont, 1987; Haeryfar and Hoskin, 2004). CD90 is composed of a single V-like immunoglobulin domain anchored by a disulfide bond between Cys 28 and Cys 104. CD90 lacks an intracellular domain but is located in the outer leaflet of lipid rafts at the cell plasma membrane allowing signaling functions by *cis*- and *trans*-interactions with G inhibitory proteins, the *Src* family kinase (SFK) members src and c-fyn, and tubulin (**Figure 1F**; Rege et al., 2006; Avalos et al., 2009; Wandel et al., 2012). Interestingly, similar to what is observed for other GPI-anchored proteins such as CD55 and CD59, CD90 could be shed by specific phospholipases (i.e., PI-PLC or PLC-β) thus allowing

cell to cell transfer, however, the physiological relevance of this process remains to be discovered (Haeryfar and Hoskin, 2004).

Common and distinct cellular CD90 expression patterns are observed in mouse and human. CD90 mRNA is highly expressed in nervous and olfactory systems, and skin tissues in both species. However, high CD90 mRNA expression is only found in mouse spleen and thymus (**Figure 1D**). In the nervous system, CD90 protein expression is observed mainly in neurons but also in some glial cells in vertebrates (**Figure 1E**). Recently, CD90 has been touted as a stem cell marker in various tissues such as in hematopoietic stem cells used in combination with the CD34 marker but also in hepatic, keratinocyte and mesenchymal stem cells (Kumar et al., 2016). Distinct cellular distributions of CD90 protein expression are observed in mouse (i.e., thymocytes and peripheral T cells) and human (i.e., endothelial cells and smooth muscle cells) (Rege and Hagood, 2006; Barker and Hagood, 2009; Bradley et al., 2009; Leyton and Hagood, 2014). Another important difference between the two species is the existence of two distinct murine isoforms CD90.1 and CD90.2 that differ at the residue 108 (Arg or Gln, respectively) whereas only one isoform is described in human with a histidine at position 108 (Bradley et al., 2009).

Several functions of CD90 have been described so far in physiological and pathological processes (**Figure 1F**). Most of these functions involve CD90 interactions with ligands such as integrins αv/β3, αx/β2, syndecan-4, CD90 itself, and CD97 (Wandel et al., 2012; Kong et al., 2013; Leyton and Hagood, 2014). CD90 plays a role in cell-cell and cell-matrix interactions, with specific implications in the regulation of axon growth and nerve regeneration, T cell activation and apoptosis, leukocytes and melanoma cell adhesion and migration, fibroblast proliferation and migration in wound healing, inflammation and fibrosis. These functions were already extensively reviewed in Rege and Hagood (2006); Barker and Hagood (2009); Bradley et al. (2009); Leyton and Hagood (2014), and will not be developed further here. Rather, we will focus on CD90 expression and functions in cancers.

DIVERSE ROLES OF CD90 IN CANCERS

CD90 Expression in Various Cancer Types

CD90 mRNA and protein expression was reported in several cancer types including liver, myeloid, skin, and brain (**Figure 2A**). According to The Cancer Genome Atlas (TCGA), CD90 transcripts were predominantly found in brain, kidney, and pancreatic tumors (**Figure 2B**). CD90 mRNA and protein were detected in glioma/GBM specimens (Wikstrand et al., 1985) and immortalized glioma/GBM cell lines (Kemshead et al., 1982; Seeger et al., 1982; Hurwitz et al., 1983; Wikstrand et al., 1983; Rettig et al., 1986). In the past few years, CD90 has been considered as a human GBM stem cell (GSC) marker (Liu et al., 2006; Kang and Kang, 2007; Tomuleasa et al., 2010; He et al., 2012; Nitta et al., 2015). CD90 is also expressed in GBM-associated stromal cells (GASCs) (Clavreul et al., 2012) and mesenchymal stem cell-like pericytes (Ochs et al., 2013), thereby reflecting GBM cellular heterogeneity. We recently demonstrated that CD90 expression is not only restricted to GBM stem-like cells but is also observed in more differentiated GBM cells (primary adherent lines) and in freshly dissociated GBM specimens (Avril et al., 2017a). Using the recent single-cell RNA sequencing datasets from stem-like and no-stem GBM cells and tumor migrating cells (Darmanis et al., 2017; Cook et al., 2018), we confirm herein that CD90 is expressed in tumor cells from the cancer site but also in migrating tumor cells, tumor-associated endothelial cells, and neighboring neuronal cells (**Figure 2C**). CD90 expression in kidney cancers is currently controversial. Primary cell lines and tumor stem cells from pediatric Wilms' tumors and metastatic renal tumors express CD90 (Pode-Shakked et al., 2009; Royer-Pokora et al., 2010; Khan et al., 2016) as observed in renal tumor-associated endothelial cells (Mesri et al., 2013). CD90 is also highly expressed in renal cell carcinoma tumor-initiating cells characterized by CD105 expression (Bussolati et al., 2008; Khan et al., 2016). Nevertheless, CD90 expression could not be found in CSCs derived from patients with clear cell renal cell carcinoma (Galleggiante et al., 2014). The CD90 protein is expressed in almost all the pancreatic adenocarcinoma (PDAC) ($n = 98$) and its metastatic forms tested by tissues microarray (Zhu et al., 2014), not only in tumors cells but also in stromal cells, including fibroblasts, and vascular endothelial cells. In addition, CD90 was extensively studied in liver cancers. Almost no CD90 expressing cells are present in disease-free or in cirrhotic livers, whereas a significantly higher expression is found in hepatocellular carcinoma (HCC) cells (Yang et al., 2008; Sukowati et al., 2013). CD90 protein is also found in esophageal squamous cell carcinomas mainly in primary tumors and immortalized/primary cell lines (Tang et al., 2013). Overexpression of CD90 is also detected in prostate cancer. Indeed immunohistochemical analysis of prostate cancer samples showed distinct and differential overexpression of CD90 in cancer-associated stroma compared with non-cancer tissue stroma (True et al., 2010).

CD90 Somatic Mutations in Cancers

Mutagenesis is often associated with carcinogenesis. Protein expression or functions could be altered by mutations leading in turn to an oncogenic process. No study has yet reported mutations in the *CD90* gene or CD90-associated related regulatory elements in any of cancer types (Kumar et al., 2016). According to the "Catalog Of Somatic Mutations In Cancer" (COSMIC; $n = 47210$ samples) and cBioPortal for Cancer Genomics ($n = 52770$ samples), the mutation frequency of CD90 in cancer is very low (0.001% with 51 and 54 mutations according to COSMIC and cBioPortal, respectively) (Cerami et al., 2012; Tate et al., 2018). Most of the CD90 mutations are missense (60.8% and 92.6% according to COSMIC and cBioPortal, respectively) and synonymous (35.3%, COSMIC) substitutions mainly found in intestine, lung, and skin cancers (**Figure 2D**). Only three mutations leading to sequence frameshift were detected in neuroblastoma and renal cancers. In addition, one RGS12/CD90 fusion was detected in a breast invasive lobular carcinoma. Further studies are needed to understand how these

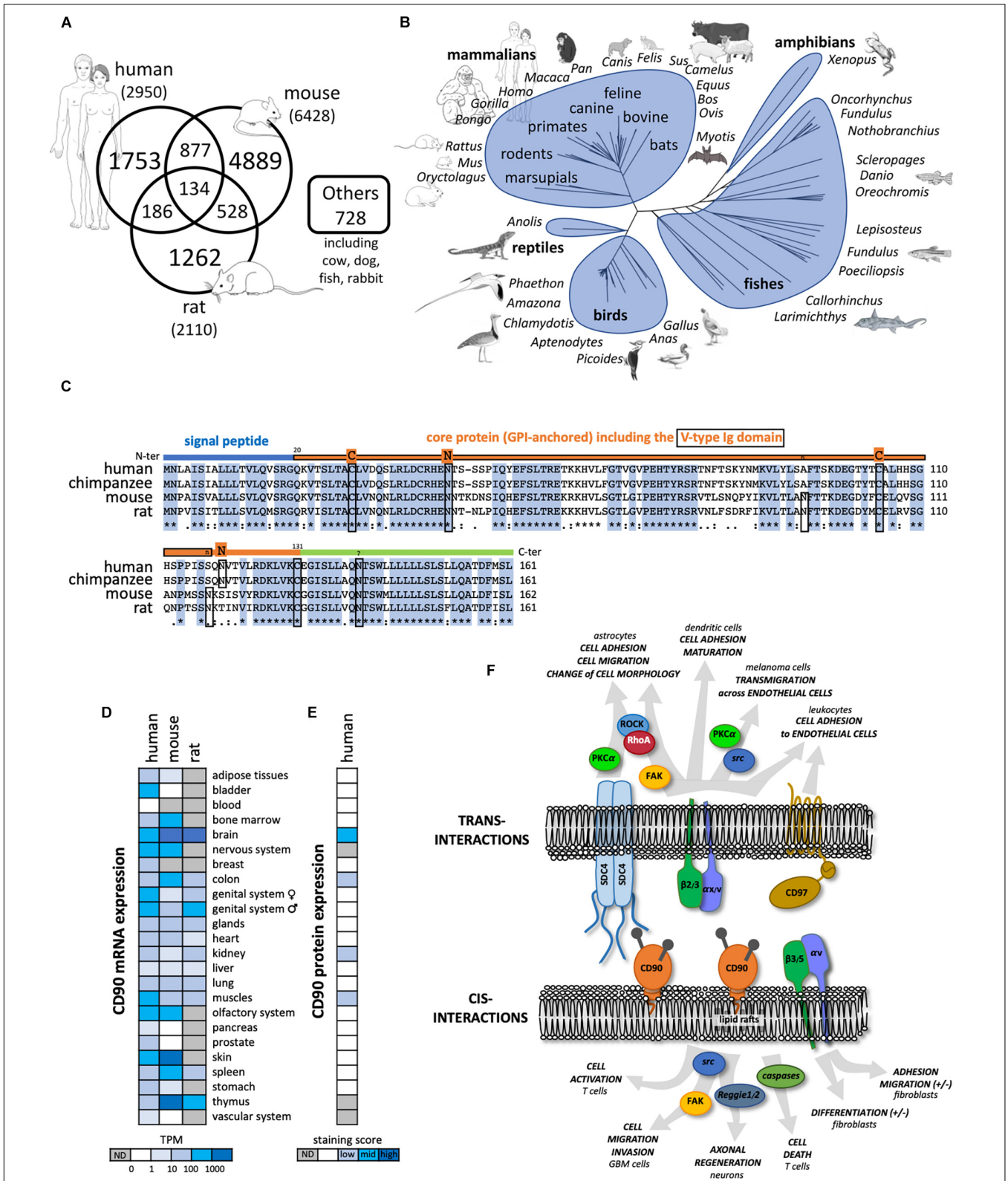

FIGURE 1 | General features of CD90 molecule. **(A)** Number of publications until November 2018 referring to CD90 according to the different species collected in Pubmed (https://www.ncbi.nlm.nih.gov/pubmed). **(B)** Tree representing the evolution of CD90 proteins among vertebrates. **(C)** The CD90 protein sequences from human, chimpanzee, mouse, and rat were aligned showing a highly conserved domains. The main features of the protein including the signal peptide (blue line), the

(Continued)

FIGURE 1 | Continued
V-type Ig domain (framed orange line), the N-glycosylation sites (n in rodents and N in primates), and the cysteines involved in the di-sulfite bond **(C)** are represented. **(D)** CD90 mRNA expression patterns in normal tissues from human, mouse and rat were analyzed using the EMBL-EBI Expression Atlas (https://www.ebi.ac.uk/gxa/home). **(E)** CD90 protein expression patterns from human normal tissues were tested using the Human Protein Atlas (https://www.proteinatlas.org/). **(F)** CD90 signaling partners and ligands interacting in *cis* and *trans* were summarized including their involvement in different functions and cell types.

mutations could impact on CD90 functions and to clarify the potential roles of these mutations in cancers.

CD90 as a Cancer Stem Cell Marker

The cancer stem cell (CSC) concept has been proposed four decades ago, and states that tumor development is driven by a specialized cell subset, characterized by self-renewing, multipotent, and tumor-initiating properties (Batlle and Clevers, 2017). In recent years, the role of CD90 was extensively studied in CSCs (Shaikh et al., 2016). The ability to form tumors *in vivo* in immunodeficient mice is considered to be one of the most important properties of CSCs. CD90+ tumor cells, considered as CSCs, from several cancers, i.e., HCC (Yang et al., 2008), gastric cancers (Jiang et al., 2012) and esophageal squamous cell carcinomas (Tang et al., 2013) were reported to form tumors in immunodeficient mice after injection of a very small amount of cells in contrast to CD90 negative counterparts. Another important feature of CSCs is their ability to grow *in vitro* as spheroids in serum-free medium. This feature has been recapitulated using CD90 expressing cells obtained from esophageal squamous cell carcinomas (Tang et al., 2013), gastric cancers (Jiang et al., 2012), gliomas (Kang and Kang, 2007; He et al., 2012), and lung carcinomas (Wang et al., 2013). Taken together, these studies identify CD90 as a potential CSC marker in many types of cancers. However, we have recently demonstrated in GBM that CD90 is not only a stem marker, as its expression is also observed in more differentiated GBM cells (Avril et al., 2017a).

CD90 as a Tumor Suppressive Molecule or a Prognostic Marker in Cancers

The prognostic role of CD90 is dependent on the cancer type. In GBM patients, high expression of CD90 in tumor specimens is associated with invasive features as demonstrated by imaging techniques (Avril et al., 2017a). These imaging features were previously linked to shorter patients' survival (Colen et al., 2014). Therefore, we proposed that CD90 expression could represent a novel stratification tool for screening patients with highly invasive tumors that could be treated with dasatinib, a SFK inhibitor. Moreover, dasatinib could not only impair the adhesion/migration of $CD90^{high}$ differentiated tumor cells but also the proliferation of $CD90^{high}$ GSCs, thereby increasing its therapeutic potential in $CD90^{high}$ tumors (Avril et al., 2017a). In hepatoblastoma, increased expression of CD90 is significantly correlated with advanced stages of the disease, poor response to treatment and lower overall survival (Bahnassy et al., 2015). CD90 overexpression was also identified as a poor prognostic marker in acute myeloid leukemia (Buccisano et al., 2004) and HCC (Lu et al., 2011). In contrast, CD90 was also shown to exert tumor suppressor functions in several others cancers, as its downregulation is associated with poor prognosis, disease progression in ovarian adenocarcinoma (Gabra et al., 1996; Abeysinghe et al., 2003), neuroblastoma (Fiegel et al., 2008) and nasopharyngeal carcinoma predominantly observed in metastatic tumor cells in invaded lymph nodes (Lung et al., 2005). CD90 inactivation is found associated with hypermethylation of the *CD90* gene promoter in CD90 negative nasopharyngeal carcinoma cell lines. Furthermore, induction of CD90 expression in nasopharyngeal carcinoma and ovarian cell lines leads to inhibition of tumor growth *in vitro* and *in vivo*, respectively (Abeysinghe et al., 2003; Lung et al., 2005). Overall, these observations illustrate the ambivalence of CD90 functions with either pro- or anti-tumoral properties depending on the cancer type.

CD90 Regulates Tumor Migration and Metastasis

Tumor invasion/migration is one of cancer hallmarks that drives to tumor dissemination leading to disease aggravation. To spread within the tissues, tumor cells use migration mechanisms that are similar to those occurring in physiological processes, including mesenchymal, amoeboid single migration or collective movements, depending on the cancer type (Friedl and Wolf, 2003; Odenthal et al., 2016). Recent studies demonstrated high invasive and metastatic capacities of CD90 expressing cells in several cancers. Indeed, NOD/SCID mice implanted subcutaneously with HCC tumor cells expressing both CD90 and CXCR4 developed distal metastatic tumors (Zhu et al., 2015). Similarly, the high metastatic capacity of CD90 and EpCAM expressing cells from primary HCC has also been observed after subcutaneously injection in immune-deficient mice (Yamashita et al., 2013). These cells have the capacity to invade surrounding tissues, to form spheroids *in vitro* and to exhibit high expression of TWIST1 and TWIST2, two important transcription factors involved in activation of Epithelial to Mesenchymal Transition (EMT) process. In addition, the presence of CD90 positive cells in HCC patients was also associated with a higher incidence of distant organ metastasis (usually occurring in one third of HCC patients) including lung, bone and adrenal gland; within 2 years after surgery (Yamashita et al., 2013). In a recent study, we demonstrated the critical role of CD90 in GBM migration/invasion mainly through the activation of SRC signaling. The same study demonstrated CD90 association with a cell adhesion/migration gene signature and with multifocal/multicentric MRI features in GBM patients. Importantly, this adhesion/migration profile is also found in other CD90-expressing tumors such as colonic, pancreatic and ovarian cancers (**Figure 2E**). Moreover, orthotopic xenografts

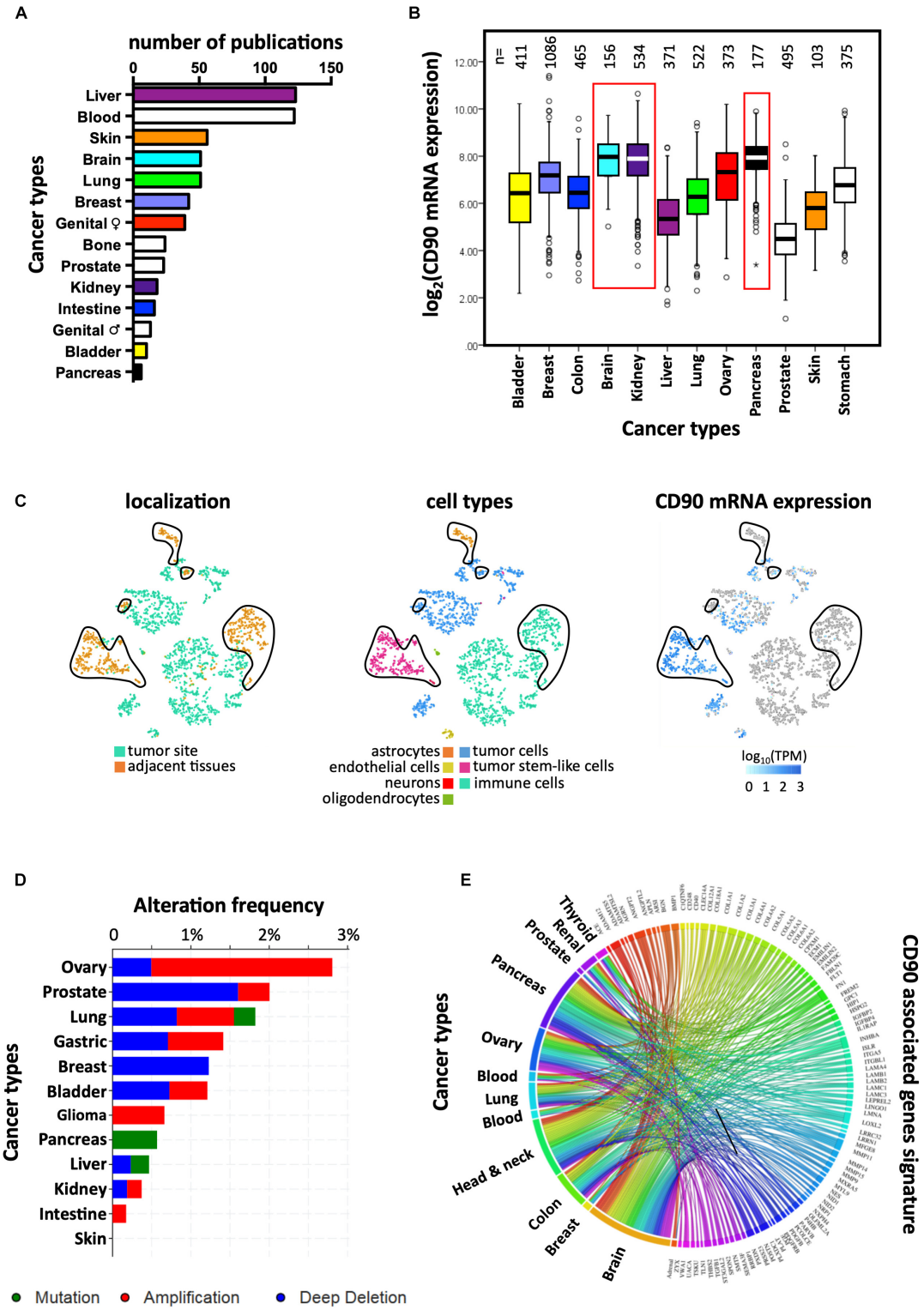

FIGURE 2 | Links between CD90 and cancers. **(A)** Articles reporting CD90 in various human cancer types were collected using Pubmed and their distribution per cancer type is presented. **(B)** CD90 mRNA expression was analyzed among the various cancer types using the TCGA resource. The corresponding number of tumor specimens tested are indicated on the top of the graph. **(C)** CD90 mRNA expression was analyzed in the single cell RNA sequencing dataset from GBM specimens (Darmanis et al., 2017) using the EMBL-EBI Single Cell Expression Atlas (https://www.ebi.ac.uk/gxa/sc/home). Cell localization and cell types are also represented. **(D)** Frequency of *CD90* gene alterations including mutations, gene amplification and deletion was analyzed among the different cancer types. **(E)** CD90 associated gene signature obtained from GBM specimens (Avril et al., 2017a) was tested in others cancer types using CancerMA tool (Feichtinger et al., 2012).

revealed that CD90 expression induced invasive phenotypes *in vivo* that could be inhibited by dasatinib (Avril et al., 2017a). In melanoma, CD90 expressing endothelial cells are mainly associated with highly metastatic tumors (Ohga et al., 2012). CD90 also mediates adhesion of melanoma cells to activated human endothelial cells via its interaction with the αv/β3 integrin on the tumor cells *in vitro* (Saalbach et al., 2005). Furthermore, expression of αv/β3 integrin (CD51/CD61), one of the CD90 ligands, in melanoma cells is associated with tumor progression and metastases formation (Ohga et al., 2012). As VEGF and TNFα induce the expression of CD90 in endothelial cells, it has been shown that mice lacking CD90 showed markedly diminished experimental lung metastasis after injection of B16/F10 melanoma cells compared to wild-type controls (Schubert et al., 2013). Interestingly, a subpopulation of breast cancer cell line MDA-MB-231 expressing CD90 and CD105, exhibits mesenchymal stem cell-like characteristics such as high migratory capacity as compared to the parental and CD90/CD105 negative cells (Wang et al., 2015). Remarkably, in breast tumor specimens, tumor cells that express both CD90 and CD44 are confined to the periphery of the tumor, representing the tumor invasive front (Donnenberg et al., 2010).

EMERGING ROLES OF THE UNFOLDED PROTEIN RESPONSE IN CANCERS: A NOVEL LINK BETWEEN CD90 AND ER STRESS ?

During tumor invasion/migration, dramatic changes occur in cells present within the compact tumor core to become single migrating cells, these transformations are described as the EMT throughout which, cells lose their cell-cell junctions, change their morphology and modify their functions leading to cell trans/de-differentiation (Friedl and Wolf, 2003; Thiery et al., 2009). Tumor development and aggressiveness including invasion and EMT were recently linked to Endoplasmic Reticulum (ER) stress signaling (Dejeans et al., 2015; Urra et al., 2016), a topic that we will further document in the next sections. Since both the signaling response triggered to cope with ER stress [also named Unfolded Protein Response (UPR)] and CD90 expression promote tumor migration, we hypothesize that CD90 and the UPR could be somehow functionally linked to control tumor cell invasive.

An Overview on UPR and Its Sensors

Despite an elaborate network of chaperones, foldases and proteins involved in the quality control of newly synthesized proteins, the ER capacity for protein synthesis and folding can be overwhelmed upon various physiological and pathological conditions, causing an accumulation on misfolded proteins into the ER and a cellular stress called ER stress. To cope with ER stress, cells activate an adaptive signaling pathway named the UPR. The UPR activates a cascade of signals leading to the attenuation of mRNA translation and to the transcriptional increase of genes whose products are involved in ER protein folding, ER protein quality control, ER-associated degradation and protein secretion. If ER stress persists, the UPR signaling shifts from adaptive to apoptotic signals thus leading to the tumor cell death (Chevet et al., 2015; Avril et al., 2017b). The UPR activation relies on 3 ER resident proteins/sensors ATF6α, IRE1α (for Inositol-Requiring Enzyme 1 alpha; referred to as IRE1 hereafter) and PERK. Initially, it was postulated that the activation of these three sensors was controlled by the ER resident chaperone GRP78/BIP and misfolded proteins themselves, thoroughly reviewed in Chevet et al. (2015); Urra et al. (2016); Avril et al. (2017b). More recently, novel mechanisms and actors of ER stress sensors activation have been described (Rojas-Rivera et al., 2018) such as the involvement of the ATP binding pocket of BIP (Carrara et al., 2015; Kopp et al., 2018); the involvement of the chaperones ERDJ4 (Amin-Wetzel et al., 2017) and HSP47 (Sepulveda et al., 2018), the ER oxidoreductase PDIA6 (Eletto et al., 2014; Groenendyk et al., 2014), and the other protein disulfide isomerase PDIA5 (Higa et al., 2014). During cancer development, tumor cells are exposed to intrinsic challenges (related to activation of their oncogenic program or aneuploidy) and to extrinsic stresses (related to nutrient and oxygen deprivation, but also anti-cancer treatments as such irradiation or chemotherapy), which lead to an altered balance between protein folding demand and the capacity of transforming cells to cope with this, thus driving ER stress (Chevet et al., 2015; Urra et al., 2016; Avril et al., 2017b; Galmiche et al., 2017). UPR sensors have been largely studied in regard to cancer diseases (**Figure 3A**). A strong involvement of one of these UPR sensors, IRE1, has been recently reported in GBM biology (Drogat et al., 2007; Auf et al., 2010; Dejeans et al., 2012; Pluquet et al., 2013; Jabouille et al., 2015; Obacz et al., 2017; Lhomond et al., 2018) and will be now presented.

IRE1 Activation and Down-Stream Signaling Pathways

IRE1 dimerizes/oligomerizes upon ER stress. This leads to its *trans*-autophosphorylation which in turn induces a conformational change to activate IRE1 endoribonuclease domain (RNase) (Chevet et al., 2015). The activation of IRE1 RNase triggers two distinct signaling pathways that lead to (i) the non-conventional splicing of the XBP1 mRNA into a novel mRNA encoding a transcription factor XBP1s; and (ii) the degradation of RNA (also called RIDD for regulated IRE1-dependent decay of RNA). XBP1s is a transcription factor that controls the expression of genes involved in protein folding, secretion, ERAD, and lipid synthesis (Chevet et al., 2015). On the other hand, RIDD targets mRNA, ribosomal RNA and microRNAs. Importantly, the selectivity of IRE1 RNase activity is highly dependent on its oligomerization state; a concept still debated as to its specificity and application (Chevet et al., 2015). IRE1 activation has also been shown to lead to c-Jun N-terminal protein kinase (JNK) phosphorylation through either the recruitment of TRAF2 (Urano et al., 2000) or the cleavage of miR17 (Lerner et al., 2012).

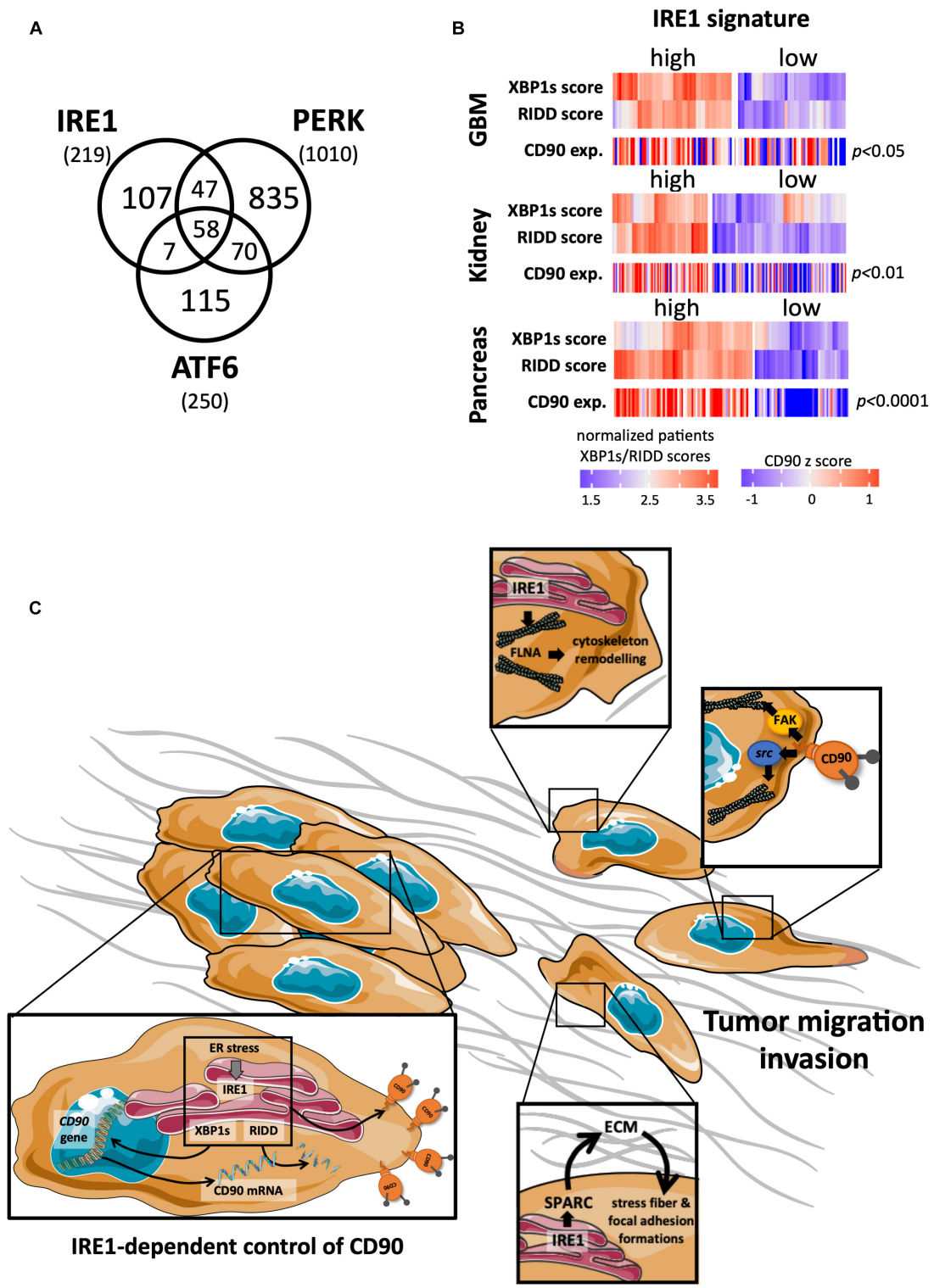

FIGURE 3 | Links between CD90 and IRE1 activity in cancers. **(A)** Articles describing a role for the ER stress sensors IRE1, PERK, and ATF6 in human cancer disease were collected using Pubmed and their distribution represented per sensor type. **(B)** CD90 mRNA expression was analyzed according to IRE1 activity from GBM, kidney and pancreas cancers (TCGA resources). Cancer patients were classified in regard to IRE1 and XBP1s/RIDD activities as described in Lhomond et al. (2018). **(C)** A schematic representation of the possible CD90 regulation by IRE1 is presented.

A Key Role of IRE1 in GBM Pathology

One of the central hallmarks of GBM is the diffuse infiltration of tumor cells into the cerebral neighboring parenchyma (Louis et al., 2007), making a complete tumor resection almost impossible (Zhong et al., 2010; Vehlow and Cordes, 2013). Interestingly, inhibition of IRE1 reduces GBM growth *in vivo* (Drogat et al., 2007; Auf et al., 2010) but alters tumor cell migration/invasion properties (Dejeans et al., 2012; Jabouille et al., 2015), acting for instance on SPARC expression, a molecule associated with the extracellular matrix (Dejeans et al., 2012). Furthermore IRE1, through its dual XBP1s and RIDD activities, exerts antagonistic effects on GBM aggressiveness influencing both tumor invasion, neo-angiogenesis and inflammation (Lhomond et al., 2018). Remarkably, GBM patients bearing tumors characterized by high IRE1 activity (and more precisely high XBP1s) exhibit a worse prognosis and display increased immune infiltration, angiogenesis and migration markers. This also opens interesting perspectives of connections between IRE1 activity and the growth and the invasion of GBM cells; however, further studies are required to understand how IRE1 downstream signaling impacts on these features.

Possible Links Between IRE1 and CD90 in Controlling GBM Cell Migration/Invasion

As our two recent studies independently demonstrated that CD90 and IRE1 could regulate GBM migration/invasion features, specific functional connections between these two proteins were considered herein. For instance, when tumors developed in mouse brain in our orthotopic mouse model, similar features of tumor infiltration, i.e., small but highly invasive tumors have been observed in CD90 expressing (Avril et al., 2017a) and IRE1 defective (Dominant Negative, DN; Auf et al., 2010) U87 cells. Due to its RNase activity, one could speculate that a functional regulatory link might exist between IRE1 and CD90 which in turn could therefore impact on GBM CD90-dependent migration. Ongoing studies from our laboratory aim at directly investigating the link between CD90 and IRE1 activity. Preliminary data indicate that ER stress inducers (such as tunicamycin, thapsigargin, and dithiothreitol) decrease the expression of cell surface CD90 in GBM cells. Intriguingly, transient expression of IRE1 defective form (DN) in U251 cells also decreased membrane CD90 expression, underlining a complex regulatory mechanism occurring between ER stress sensors (including IRE1) and CD90. Importantly, applying our recent classification of GBM patients according to IRE1 gene signature on the GBM TCGA cohort, we observed that tumors with high IRE1 activity expressed higher levels CD90 mRNA than tumors exhibiting low IRE1 activity. Importantly, this could be applied to others cancer types including renal and pancreas (**Figure 3B**). Furthermore, a CD90 associated gene signature described in Avril et al. (2017a) was also associated with IRE1 activity. Overall, these observations highlight the potential effect of IRE activity on CD90 expression and its potential role in functions linked to tumor migration/invasion. Interestingly, IRE1 has already been associated with molecules involved in cell migration, i.e., by controlling SPARC expression (Dejeans et al., 2012) and interacting directly with filamin A (Urra et al., 2018). Further functional and molecular studies are needed to better understand the connections between CD90 and IRE1 in cancer development, and in particular migration and invasion (**Figure 3C**).

CONCLUSION

Increasing evidence supports the importance of CD90 in cancer development. CD90 has been mainly considered as a useful CSC marker in various cancer types such as kidney, brain, and liver. However, even despite the absence of intracellular domain, CD90 is also able to transmit intracellular signals that lead to the activation of tumor cell migration/invasion program in liver and lung cancers, in GBM and in melanoma. In contrast, CD90 is described as a tumor suppressor molecule in nasopharyngeal carcinoma. Although further studies are required to clearly demonstrate the connections between ER stress signaling and CD90 expression, initial transcriptome analyses from cancer patients indicate that CD90 expression appears to be dependent on the activation of the UPR, a key event in various oncological clinical settings including brain, kidney, and pancreatic cancers. These different elements underline the complexity of CD90 functions in cancer, depending on both the cellular context and on the tumor microenvironment. Future studies will lead to the better understanding of CD90 regulation and functions adding to the information already available such as the CD90 mutation spectrum as seen in COSMIC/cBioPortal; the IRE1-controlled CD90 tumor expression and functions with a clarification of the involvement of the IRE1 downstream signaling pathways XBP1s and/or RIDD branches; and the relevance of cleaved CD90 released in the tumor microenvironment.

AUTHOR CONTRIBUTIONS

CS wrote the sections of the manuscript. KV and AC organized the database and performed the statistical analysis. EC and TA contributed to conception and design of the review. TA wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

Abeysinghe, H. R., Cao, Q., Xu, J., Pollock, S., Veyberman, Y., Guckert, N. L., et al. (2003). THY1 expression is associated with tumor suppression of human ovarian cancer. *Cancer Genet. Cytogenet.* 143, 125–132. doi: 10.1016/s0165-4608(02)00855-5

Amin-Wetzel, N., Saunders, R. A., Kamphuis, M. J., Rato, C., Preissler, S., Harding, H. P., et al. (2017). A J-protein co-chaperone recruits BiP to monomerize IRE1 and repress the unfolded protein response. *Cell* 171, 1625.e13–1637.e13. doi: 10.1016/j.cell.2017.10.040

Auf, G., Jabouille, A., Guerit, S., Pineau, R., Delugin, M., Bouchecareilh, M., et al. (2010). Inositol-requiring enzyme 1alpha is a key regulator of angiogenesis and invasion in malignant glioma. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15553–15558. doi: 10.1073/pnas.0914072107

Avalos, A. M., Valdivia, A. D., Munoz, N., Herrera-Molina, R., Tapia, J. C., Lavandero, S., et al. (2009). Neuronal Thy-1 induces astrocyte adhesion by engaging syndecan-4 in a cooperative interaction with alphavbeta3 integrin that activates PKCalpha and RhoA. *J. Cell Sci.* 122(Pt 19), 3462–3471. doi: 10.1242/jcs.034827

Avril, T., Etcheverry, A., Pineau, R., Obacz, J., Jegou, G., Jouan, F., et al. (2017a). CD90 expression controls migration and predicts dasatinib response in Glioblastoma. *Clin. Cancer Res.* 23, 7360–7374. doi: 10.1158/1078-0432.CCR-17-1549

Avril, T., Vauleon, E., and Chevet, E. (2017b). Endoplasmic reticulum stress signaling and chemotherapy resistance in solid cancers. *Oncogenesis* 6:e373. doi: 10.1038/oncsis.2017.72

Bahnassy, A. A., Fawzy, M., El-Wakil, M., Zekri, A.-R. N., Abdel-Sayed, A., and Sheta, M. (2015). Aberrant expression of cancer stem cell markers (CD44, CD90, and CD133) contributes to disease progression and reduced survival in hepatoblastoma patients: 4-year survival data. *Transl. Res.* 165, 396–406. doi: 10.1016/j.trsl.2014.07.009

Barclay, A. N., Letarte-Muirhead, M., Williams, A. F., and Faulkes, R. A. (1976). Chemical characterisation of the Thy-1 glycoproteins from the membranes of rat thymocytes and brain. *Nature* 263, 563–567. doi: 10.1038/263563a0

Barker, T. H., and Hagood, J. S. (2009). Getting a grip on Thy-1 signaling. *Biochim. Biophys. Acta* 1793, 921–923. doi: 10.1016/j.bbamcr.2008.10.004

Batlle, E., and Clevers, H. (2017). Cancer stem cells revisited. *Nat. Med.* 23, 1124–1134. doi: 10.1038/nm.4409

Bradley, J. E., Ramirez, G., and Hagood, J. S. (2009). Roles and regulation of Thy-1, a context-dependent modulator of cell phenotype. *BioFactors* 35, 258–265. doi: 10.1002/biof.41

Buccisano, F., Rossi, F. M., Venditti, A., Del Poeta, G., Cox, M. C., Abbruzzese, E., et al. (2004). CD90/Thy-1 is preferentially expressed on blast cells of high risk acute myeloid leukaemias. *Br. J. Haematol.* 125, 203–212. doi: 10.1111/j.1365-2141.2004.04883.x

Bussolati, B., Bruno, S., Grange, C., Ferrando, U., and Camussi, G. (2008). Identification of a tumor-initiating stem cell population in human renal carcinomas. *FASEB J.* 22, 3696–3705. doi: 10.1096/fj.08-102590

Carrara, M., Prischi, F., Nowak, P. R., Kopp, M. C., and Ali, M. M. (2015). Noncanonical binding of BiP ATPase domain to Ire1 and Perk is dissociated by unfolded protein CH1 to initiate ER stress signaling. *eLife* 4:e03522. doi: 10.7554/eLife.03522

Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., et al. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* 2, 401–404. doi: 10.1158/2159-8290.CD-12-0095

Chevet, E., Hetz, C., and Samali, A. (2015). Endoplasmic reticulum stress-activated cell reprogramming in oncogenesis. *Cancer Discov.* 5, 586–597. doi: 10.1158/2159-8290.CD-14-1490

Clavreul, A., Etcheverry, A., Chassevent, A., Quillien, V., Avril, T., Jourdan, M. L., et al. (2012). Isolation of a new cell population in the glioblastoma microenvironment. *J. Neurooncol.* 106, 493–504. doi: 10.1007/s11060-011-0701-7

Colen, R. R., Vangel, M., Wang, J., Gutman, D. A., Hwang, S. N., Wintermark, M., et al. (2014). Imaging genomic mapping of an invasive MRI phenotype predicts patient outcome and metabolic dysfunction: a TCGA glioma phenotype research group project. *BMC Med. Genomics* 7:30. doi: 10.1186/1755-8794-7-30

Cook, C. E., Lopez, R., Stroe, O., Cochrane, G., Brooksbank, C., Birney, E., et al. (2018). The European bioinformatics institute in 2018: tools, infrastructure and training. *Nucleic Acids Res.* 47, D15–D22. doi: 10.1093/nar/gky1124

Cooper, E. L., and Mansour, M. H. (1989). Distribution of Thy-1 in invertebrates and ectothermic vertebrates. *Immunol. Ser.* 45, 197–219.

Darmanis, S., Sloan, S. A., Croote, D., Mignardi, M., Chernikova, S., Samghababi, P., et al. (2017). Single-Cell RNA-Seq analysis of infiltrating neoplastic cells at the migrating front of human Glioblastoma. *Cell Rep.* 21, 1399–1410. doi: 10.1016/j.celrep.2017.10.030

Dejeans, N., Barroso, K., Fernandez-Zapico, M. E., Samali, A., and Chevet, E. (2015). Novel roles of the unfolded protein response in the control of tumor development and aggressiveness. *Semin. Cancer Biol.* 33, 67–73. doi: 10.1016/j.semcancer.2015.04.007

Dejeans, N., Pluquet, O., Lhomond, S., Grise, F., Bouchecareilh, M., Juin, A., et al. (2012). Autocrine control of glioma cells adhesion and migration through IRE1alpha-mediated cleavage of SPARC mRNA. *J. Cell Sci.* 125(Pt 18), 4278–4287. doi: 10.1242/jcs.099291

Donnenberg, V. S., Donnenberg, A. D., Zimmerlin, L., Landreneau, R. J., Bhargava, R., Wetzel, R. A., et al. (2010). Localization of CD44 and CD90 positive cells to the invasive front of breast tumors. *Cytometry B Clin. Cytom.* 78, 287–301. doi: 10.1002/cyto.b.20530

Drogat, B., Auguste, P., Nguyen, D. T., Bouchecareilh, M., Pineau, R., Nalbantoglu, J., et al. (2007). IRE1 signaling is essential for ischemia-induced vascular endothelial growth factor-A expression and contributes to angiogenesis and tumor growth in vivo. *Cancer Res.* 67, 6700–6707. doi: 10.1158/0008-5472.can-06-3235

Eletto, D., Eletto, D., Dersh, D., Gidalevitz, T., and Argon, Y. (2014). Protein disulfide isomerase A6 controls the decay of IRE1alpha signaling via disulfide-dependent association. *Mol. Cell* 53, 562–576. doi: 10.1016/j.molcel.2014.01.004

Feichtinger, J., McFarlane, R. J., and Larcombe, L. D. (2012). CancerMA: a web-based tool for automatic meta-analysis of public cancer microarray data. *Database* 2012:bas055. doi: 10.1093/database/bas055

Feng, G., Mellor, R. H., Bernstein, M., Keller-Peck, C., Nguyen, Q. T., Wallace, M., et al. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28, 41–51. doi: 10.1016/s0896-6273(00)00084-2

Fiegel, H. C., Kaifi, J. T., Quaas, A., Varol, E., Krickhahn, A., Metzger, R., et al. (2008). Lack of Thy1 (CD90) expression in neuroblastomas is correlated with impaired survival. *Pediatr. Surg. Int.* 24, 101–105. doi: 10.1007/s00383-007-2033-4

Friedl, P., and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer* 3, 362–374. doi: 10.1038/nrc1075

Gabra, H., Watson, J. E., Taylor, K. J., Mackay, J., Leonard, R. C., Steel, C. M., et al. (1996). Definition and refinement of a region of loss of heterozygosity at 11q23.3-q24.3 in epithelial ovarian cancer associated with poor prognosis. *Cancer Res.* 56, 950–954.

Galleggiante, V., Rutigliano, M., Sallustio, F., Ribatti, D., Ditonno, P., Bettocchi, C., et al. (2014). CTR2 identifies a population of cancer cells with stem cell-like features in patients with clear cell renal cell carcinoma. *J. Urol.* 192, 1831–1841. doi: 10.1016/j.juro.2014.06.070

Galmiche, A., Sauzay, C., Chevet, E., and Pluquet, O. (2017). Role of the unfolded protein response in tumor cell characteristics and cancer outcome. *Curr. Opin. Oncol.* 29, 41–47. doi: 10.1097/cco.0000000000000339

Groenendyk, J., Peng, Z., Dudek, E., Fan, X., Mizianty, M. J., Dufey, E., et al. (2014). Interplay between the oxidoreductase PDIA6 and microRNA-322 controls the response to disrupted endoplasmic reticulum calcium homeostasis. *Sci. Signal.* 7:ra54. doi: 10.1126/scisignal.2004983

Haeryfar, S. M., and Hoskin, D. W. (2004). Thy-1: more than a mouse pan-T cell marker. *J. Immunol.* 173, 3581–3588. doi: 10.4049/jimmunol.173.6.3581

He, J., Liu, Y., Zhu, T., Zhu, J., Dimeco, F., Vescovi, A. L., et al. (2012). CD90 is identified as a candidate marker for cancer stem cells in primary high-grade gliomas using tissue microarrays. *Mol. Cell. Proteomics* 11:M111.010744. doi: 10.1074/mcp.M111.010744

Higa, A., Taouji, S., Lhomond, S., Jensen, D., Fernandez-Zapico, M. E., Simpson, J. C., et al. (2014). Endoplasmic reticulum stress-activated transcription factor ATF6alpha requires the disulfide isomerase PDIA5 to modulate chemoresistance. *Mol. Cell. Biol.* 34, 1839–1849. doi: 10.1128/MCB.01484-13

Hurwitz, E., Arnon, R., Sahar, E., and Danon, Y. (1983). A conjugate of adriamycin and monoclonal antibodies to Thy-1 antigen inhibits human neuroblastoma cells in vitro. *Ann. N. Y. Acad. Sci.* 417, 125–136. doi: 10.1111/j.1749-6632.1983.tb32857.x

Jabouille, A., Delugin, M., Pineau, R., Dubrac, A., Soulet, F., Lhomond, S., et al. (2015). Glioblastoma invasion and cooption depend on IRE1alpha endoribonuclease activity. *Oncotarget* 6, 24922–24934. doi: 10.18632/oncotarget.4679

Jiang, J., Zhang, Y., Chuai, S., Wang, Z., Zheng, D., Xu, F., et al. (2012). Trastuzumab (herceptin) targets gastric cancer stem cells characterized by CD90 phenotype. *Oncogene* 31, 671–682. doi: 10.1038/onc.2011.282

Kang, M.-K., and Kang, S.-K. (2007). Tumorigenesis of chemotherapeutic drug-resistant cancer stem-like cells in brain glioma. *Stem Cells Dev.* 16, 837–847.

Kemshead, J. T., Ritter, M. A., Cotmore, S. F., and Greaves, M. F. (1982). Human Thy-1: expression on the cell surface of neuronal and glial cells. *Brain Res.* 236, 451–461. doi: 10.1016/0006-8993(82)90727-2

Khan, M. I., Czarnecka, A. M., Lewicki, S., Helbrecht, I., Brodaczewska, K., Koch, I., et al. (2016). Comparative gene expression profiling of primary and metastatic renal cell carcinoma stem cell-like cancer cells. *PLoS One* 11:e0165718. doi: 10.1371/journal.pone.0165718

Kong, M., Munoz, N., Valdivia, A., Alvarez, A., Herrera-Molina, R., Cardenas, A., et al. (2013). Thy-1-mediated cell-cell contact induces astrocyte migration through the engagement of alphaVbeta3 integrin and syndecan-4. *Biochim. Biophys. Acta* 1833, 1409–1420. doi: 10.1016/j.bbamcr.2013.02.013

Kopp, M. C., Nowak, P. R., Larburu, N., Adams, C. J., and Ali, M. M. (2018). In vitro FRET analysis of IRE1 and BiP association and dissociation upon endoplasmic reticulum stress. *eLife* 7:e30257. doi: 10.7554/eLife.30257

Kumar, A., Bhanja, A., Bhattacharyya, J., and Jaganathan, B. G. (2016). Multiple roles of CD90 in cancer. *Tumour Biol.* 37, 11611–11622. doi: 10.1007/s13277-016-5112-0

Lerner, A. G., Upton, J. P., Praveen, P. V., Ghosh, R., Nakagawa, Y., Igbaria, A., et al. (2012). IRE1alpha induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. *Cell Metab.* 16, 250–264. doi: 10.1016/j.cmet.2012.07.007

Leyton, L., and Hagood, J. S. (2014). Thy-1 modulates neurological cell-cell and cell-matrix interactions through multiple molecular interactions. *Adv. Neurobiol.* 8, 3–20. doi: 10.1007/978-1-4614-8090-7_1

Lhomond, S., Avril, T., Dejeans, N., McMahon, M., Pineau, R., Papadodima, O., et al. (2018). Antagonistic IRE1 RNase functions dictate glioblastoma tumor development. *EMBO Mol. Med.* 10:e7929. doi: 10.15252/emmm.201707929

Liu, G., Yuan, X., Zeng, Z., Tunici, P., Ng, H., Abdulkadir, I. R., et al. (2006). Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol. Cancer* 5:67.

Louis, D. N., Ohgaki, H., Wiestler, O. D., Cavenee, W. K., Burger, P. C., Jouvet, A., et al. (2007). The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 114, 97–109.

Lu, J.-W., Chang, J.-G., Yeh, K.-T., Chen, R.-M., Tsai, J. J. P., and Hu, R.-M. (2011). Overexpression of Thy1/CD90 in human hepatocellular carcinoma is associated with HBV infection and poor prognosis. *Acta Histochem.* 113, 833–838. doi: 10.1016/j.acthis.2011.01.001

Lung, H. L., Bangarusamy, D. K., Xie, D., Cheung, A. K. L., Cheng, Y., Kumaran, M. K., et al. (2005). THY1 is a candidate tumour suppressor gene with decreased expression in metastatic nasopharyngeal carcinoma. *Oncogene* 24, 6525–6532. doi: 10.1038/sj.onc.1208812

Mesri, M., Birse, C., Heidbrink, J., McKinnon, K., Brand, E., Bermingham, C. L., et al. (2013). Identification and characterization of angiogenesis targets through proteomic profiling of endothelial cells in human cancer tissues. *PLoS One* 8:e78885. doi: 10.1371/journal.pone.0078885

Nitta, R. T., Gholamin, S., Feroze, A. H., Agarwal, M., Cheshier, S. H., Mitra, S. S., et al. (2015). Casein kinase 2alpha regulates glioblastoma brain tumor-initiating cell growth through the beta-catenin pathway. *Oncogene* 34, 3688–3699. doi: 10.1038/onc.2014.299

Obacz, J., Avril, T., Le Reste, P. J., Urra, H., Quillien, V., Hetz, C., et al. (2017). Endoplasmic reticulum proteostasis in glioblastoma-From molecular mechanisms to therapeutic perspectives. *Sci. Signal.* 10:eaal2323. doi: 10.1126/scisignal.aal2323

Ochs, K., Sahm, F., Opitz, C. A., Lanz, T. V., Oezen, I., Couraud, P. O., et al. (2013). Immature mesenchymal stem cell-like pericytes as mediators of immunosuppression in human malignant glioma. *J. Neuroimmunol.* 265, 106–116. doi: 10.1016/j.jneuroim.2013.09.011

Odenthal, J., Takes, R., and Friedl, P. (2016). Plasticity of tumor cell invasion: governance by growth factors and cytokines. *Carcinogenesis* 37, 1117–1128.

Ohga, N., Ishikawa, S., Maishi, N., Akiyama, K., Hida, Y., Kawamoto, T., et al. (2012). Heterogeneity of tumor endothelial cells: comparison between tumor endothelial cells isolated from high- and low-metastatic tumors. *Am. J. Pathol.* 180, 1294–1307. doi: 10.1016/j.ajpath.2011.11.035

Pluquet, O., Dejeans, N., Bouchecareilh, M., Lhomond, S., Pineau, R., Higa, A., et al. (2013). Posttranscriptional regulation of PER1 underlies the oncogenic function of IREalpha. *Cancer Res.* 73, 4732–4743. doi: 10.1158/0008-5472.CAN-12-3989

Pode-Shakked, N., Metsuyanim, S., Rom-Gross, E., Mor, Y., Fridman, E., Goldstein, I., et al. (2009). Developmental tumourigenesis: NCAM as a putative marker for the malignant renal stem/progenitor cell population. *J. Cell. Mol. Med.* 13, 1792–1808. doi: 10.1111/j.1582-4934.2008.00607.x

Pont, S. (1987). Thy-1: a lymphoid cell subset marker capable of delivering an activation signal to mouse T lymphocytes. *Biochimie* 69, 315–320. doi: 10.1016/0300-9084(87)90022-8

Rege, T. A., and Hagood, J. S. (2006). Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *FASEB J.* 20, 1045–1054. doi: 10.1096/fj.05-5460rev

Rege, T. A., Pallero, M. A., Gomez, C., Grenett, H. E., Murphy-Ullrich, J. E., and Hagood, J. S. (2006). Thy-1, via its GPI anchor, modulates Src family kinase and focal adhesion kinase phosphorylation and subcellular localization, and fibroblast migration, in response to thrombospondin-1/hep I. *Exp. Cell Res.* 312, 3752–3767. doi: 10.1016/j.yexcr.2006.07.029

Reif, A. E., and Allen, J. M. (1964a). Immunological distinction of Akr thymocytes. *Nature* 203, 886–887. doi: 10.1038/203886a0

Reif, A. E., and Allen, J. M. (1964b). The Akr thymic antigen and its distribution in leukemias and nervous tissues. *J. Exp. Med.* 120, 413–433. doi: 10.1084/jem.120.3.413

Rettig, W. J., Chesa, P. G., Beresford, H. R., Feickert, H. J., Jennings, M. T., Cohen, J., et al. (1986). Differential expression of cell surface antigens and glial fibrillary acidic protein in human astrocytoma subsets. *Cancer Res.* 46(12 Pt 1), 6406–6412.

Rojas-Rivera, D., Rodriguez, D. A., Sepulveda, D., and Hetz, C. (2018). ER stress sensing mechanism: putting off the brake on UPR transducers. *Oncotarget* 9, 19461–19462.

Royer-Pokora, B., Busch, M., Beier, M., Duhme, C., de Torres, C., Mora, J., et al. (2010). Wilms tumor cells with WT1 mutations have characteristic features of mesenchymal stem cells and express molecular markers of paraxial mesoderm. *Hum. Mol. Genet.* 19, 1651–1668. doi: 10.1093/hmg/ddq042

Saalbach, A., Wetzel, A., Haustein, U.-F., Sticherling, M., Simon, J. C., and Anderegg, U. (2005). Interaction of human Thy-1 (CD 90) with the integrin alphavbeta3 (CD51/CD61): an important mechanism mediating melanoma cell adhesion to activated endothelium. *Oncogene* 24, 4710–4720. doi: 10.1038/sj.onc.1208559

Schubert, K., Gutknecht, D., Köberle, M., Anderegg, U., and Saalbach, A. (2013). Melanoma cells use Thy-1 (CD90) on endothelial cells for metastasis formation. *Am. J. Pathol.* 182, 266–276. doi: 10.1016/j.ajpath.2012.10.003

Seeger, R. C., Danon, Y. L., Rayner, S. A., and Hoover, F. (1982). Definition of a Thy-1 determinant on human neuroblastoma, glioma, sarcoma, and teratoma cells with a monoclonal antibody. *J. Immunol.* 128, 983–989.

Seki, T., Spurr, N., Obata, F., Goyert, S., Goodfellow, P., and Silver, J. (1985). The human Thy-1 gene: structure and chromosomal location. *Proc. Natl. Acad. Sci. U.S.A.* 82, 6657–6661. doi: 10.1073/pnas.82.19.6657

Sepulveda, D., Rojas-Rivera, D., Rodriguez, D. A., Groenendyk, J., Kohler, A., Lebeaupin, C., et al. (2018). Interactome screening identifies the ER luminal chaperone Hsp47 as a regulator of the unfolded protein response transducer IRE1alpha. *Mol. Cell* 69, 238.e7–252.e7. doi: 10.1016/j.molcel.2017.12.028

Shaikh, M. V., Kala, M., and Nivsarkar, M. (2016). CD90 a potential cancer stem cell marker and a therapeutic target. *Cancer Biomark.* 16, 301–307. doi: 10.3233/CBM-160590

Sukowati, C. H. C., Anfuso, B., Torre, G., Francalanci, P., Crocè, L. S., and Tiribelli, C. (2013). The expression of CD90/Thy-1 in hepatocellular carcinoma: an in vivo and in vitro study. *PLoS One* 8:e76830. doi: 10.1371/journal.pone.0076830

Tang, K. H., Dai, Y. D., Tong, M., Chan, Y. P., Kwan, P. S., Fu, L., et al. (2013). A CD90+ tumor-initiating cell population with an aggressive signature and metastatic capacity in esophageal cancer. *Cancer Res.* 73, 2322–2332. doi: 10.1158/0008-5472.CAN-12-2991

Tate, J. G., Bamford, S., Jubb, H. C., Sondka, Z., Beare, D. M., Bindal, N., et al. (2018). COSMIC: the catalogue of somatic mutations in cancer. *Nucleic Acids Res.* 47, D941–D947. doi: 10.1093/nar/gky1015

Thiery, J. P., Acloque, H., Huang, R. Y., and Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell* 139, 871–890. doi: 10.1016/j.cell.2009.11.007

Tomuleasa, C., Soritau, O., Rus-Ciuca, D., Ioani, H., Susman, S., Petrescu, M., et al. (2010). Functional and molecular characterization of glioblastoma multiforme-derived cancer stem cells. *J. BUON* 15, 583–591.

True, L. D., Zhang, H., Ye, M., Huang, C.-Y., Nelson, P. S., Haller, P. D. V., et al. (2010). CD90/THY1 is overexpressed in prostate cancer-associated fibroblasts and could serve as a cancer biomarker. *Mod. Pathol.* 23, 1346–1356. doi: 10.1038/modpathol.2010.122

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., et al. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287, 664–666. doi: 10.1126/science.287.5453.664

Urra, H., Dufey, E., Avril, T., Chevet, E., and Hetz, C. (2016). Endoplasmic reticulum stress and the hallmarks of cancer. *Trends Cancer* 2, 252–262. doi: 10.1016/j.trecan.2016.03.007

Urra, H., Henriquez, D. R., Canovas, J., Villarroel-Campos, D., Carreras-Sureda, A., Pulgar, E., et al. (2018). IRE1alpha governs cytoskeleton remodelling and cell migration through a direct interaction with filamin A. *Nat. Cell Biol.* 20, 942–953. doi: 10.1038/s41556-018-0141-0

Vehlow, A., and Cordes, N. (2013). Invasion as target for therapy of glioblastoma multiforme. *Biochim. Biophys. Acta* 1836, 236–244. doi: 10.1016/j.bbcan.2013.07.001

Wandel, E., Saalbach, A., Sittig, D., Gebhardt, C., and Aust, G. (2012). Thy-1 (CD90) is an interacting partner for CD97 on activated endothelial cells. *J. Immunol.* 188, 1442–1450. doi: 10.4049/jimmunol.1003944

Wang, P., Gao, Q., Suo, Z., Munthe, E., Solberg, S., Ma, L., et al. (2013). Identification and characterization of cells with cancer stem cell properties in human primary lung cancer cell lines. *PLoS One* 8:e57020. doi: 10.1371/journal.pone.0057020

Wang, X., Liu, Y., Zhou, K., Zhang, G., Wang, F., and Ren, J. (2015). Isolation and characterization of CD105+/CD90+ subpopulation in breast cancer MDA-MB-231 cell line. *Int. J. Clin. Exp. Pathol.* 8, 5105–5112.

Wikstrand, C. J., Bigner, S. H., and Bigner, D. D. (1983). Demonstration of complex antigenic heterogeneity in a human glioma cell line and eight derived clones by specific monoclonal antibodies. *Cancer Res.* 43, 3327–3334.

Wikstrand, C. J., Grahmann, F. C., McComb, R. D., and Bigner, D. D. (1985). Antigenic heterogeneity of human anaplastic gliomas and glioma-derived cell lines defined by monoclonal antibodies. *J. Neuropathol. Exp. Neurol.* 44, 229–241. doi: 10.1097/00005072-198505000-00002

Yamashita, T., Honda, M., Nakamoto, Y., Baba, M., Nio, K., Hara, Y., et al. (2013). Discrete nature of EpCAM+ and CD90+ cancer stem cells in human hepatocellular carcinoma. *Hepatology* 57, 1484–1497. doi: 10.1002/hep.26168

Yang, Z. F., Ho, D. W., Ng, M. N., Lau, C. K., Yu, W. C., Ngai, P., et al. (2008). Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell* 13, 153–166. doi: 10.1016/j.ccr.2008.01.013

Zhong, J., Paul, A., Kellie, S. J., and O'Neill, G. M. (2010). Mesenchymal migration as a therapeutic target in glioblastoma. *J. Oncol.* 2010:430142. doi: 10.1155/2010/430142

Zhu, J., Thakolwiboon, S., Liu, X., Zhang, M., and Lubman, D. M. (2014). Overexpression of CD90 (Thy-1) in pancreatic adenocarcinoma present in the tumor microenvironment. *PLoS One* 9:e115507. doi: 10.1371/journal.pone.0115507

Zhu, L., Zhang, W., Wang, J., and Liu, R. (2015). Evidence of CD90+CXCR4+ cells as circulating tumor stem cells in hepatocellular carcinoma. *Tumour Biol.* 36, 5353–5360. doi: 10.1007/s13277-015-3196-6

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