mg/kg, i.p.) failed to alleviate scratching behavior in mice injected with bilirubin (Figure 1—figure supplement 1D). Furthermore, bilirubin did not elicit a calcium response or induce appreciable histamine release from peritoneal mast cells (Figure 1—figure supplement 1E–F).

The Mas-related G-protein coupled receptor (Mrgpr) family of receptors is a major mediator of non-histaminergic pruritus (Han et al., 2013; Liu et al., 2012; Liu et al., 2009; Sikand et al., 2011). To test whether Mrgprs mediate bilirubin-induced pruritus, we injected mice lacking a cluster of 12 Mrgpr genes (Mrgpr-clusterD

/

or Mrgpr-cluster KO) with bilirubin (Liu et al., 2009). Mrgpr-cluster

KO animals scratched approximately 75% less than wild type (WT) mice, indicating that one or more of the 12 Mrgprs within the cluster mediates bilirubin-induced pruritus (Figure 1C).

To identify which Mrgpr is sensitive to bilirubin, we individually expressed each of the 12 Mrgprs deleted in the Mrgpr-cluster KO mouse in human embryonic kidney (HEK) 293 cells and monitored changes in intracellular calcium upon applying bilirubin. To ensure we would observe a calcium response following a true ligand-receptor interaction, we expressed the receptors in HEK293 cells stably expressing the G-protein alpha-subunit Ga15, a Ga protein that couples GPCRs to intracellular calcium stores via phospholipase C (PLC).

Among the twelve cell lines expressing an Mrgpr, only MRGPRA1-expressing cells exhibited a calcium response to bilirubin (EC50 of 145.9 mM (Alemi et al., 2013)) (Figure 2A,D). The same cells that responded to bilirubin also responded to FMRF, an MRGPRA1 agonist (Dong et al., 2001). To ensure that bilirubin initiated signaling at MRGPRA1 and not downstream, we pre-treated MRGPRA1-expressing cells with inhibitors of GPCR signaling: the PLC inhibitor U73122 or the Gaq inhibitor YM-254890. Both compounds abolished bilirubin-induced calcium responses (Figure 2B–C).

In addition to bilirubin, glucuronidated bilirubin is often upregulated in jaundice-associated itch. We assessed whether ditaurate bilirubin, a distinct but similar bilirubin derivative, could activate MRGPRA1. Indeed, ditaurate bilirubin activated MRGPRA1-expressing cells (Figure 2D). Hemin failed to activate MRGPRA1 (Figure 2D), consistent with our earlier behavioral findings in which hemin did not evoke scratching. No other Mrgpr among the 12 that we screened responded to bilirubin (Figure 2N, Figure 2—figure supplement 1).

The human MRGPRX family of receptors has functional similarities between species but have no

obvious structural homologs in rodents (Solinski et al., 2014; Zylka et al., 2003). The mouse Mrgpra family is closest in sequence homology to the human MRGPRX family (Dong et al., 2001; Lembo et al., 2002; Zhang et al., 2005). Of the four human MRGPRX receptors, only MRGPRX4-expressing cells responded to bilirubin (EC50 of 61.9 mM (Azimi et al., 2017)) (Figure 2F,I). U73122 and YM-254890 inhibited bilirubin-induced calcium responses in MRGPRX4-expressing cells just as with MRGPRA1 (Figure 2G–H). Conjugated bilirubin also activated MRGPRX4, whereas hemin had no effect (Figure 2I).

To confirm that bilirubin directly binds the identified receptors, we assayed thermophoresis of each receptor in the presence and absence of bilirubin. Thermophoresis of a molecule is affected by physical parameters such as size, charge, and solvation. By extension, the thermophoresis of one molecule is altered when it interacts with another, and can therefore be used to measure interactions between molecules (Duhr and Braun, 2006). Using this approach, we determined that bilirubin bound MRGPRA1 with a KD of 92.9 ± 15 mM and MRGPRX4 with a KD of 54.4 ± 13 mM (Figure 2E,J). Bilirubin exhibited little to no affinity for the closely related BAM8-22 receptor MRGPRC11 (Figure 2O). Hemin, which did not activate MRGPRA1 or MRGPRX4 by calcium imaging (Figure 2D, I), also did not bind MRGPRA1 or MRGPRX4 (Figure 2E,J). Conjugated bilirubin bound both MRGPRA1 and MRGPRX4, although with a lower affinity than unconjugated bilirubin (Figure 2E,J).

To make certain that bilirubin activates MRGPRA1 and MRGPRX4 upon binding, we measured exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), one of the first events in GPCR signaling. Bilirubin increased GTP binding to MRGPRA1 and MRGPRX4 membrane complexes, but not to MRGPRC11 (Figure 2K). To confirm that bilirubin activates MRGPRA1 in vivo to trigger itch, we generated an Mrgpra1 (A1 KO) knockout mouse line using CRISPR-Cas9 (Jinek et al., 2012) (Figure 2—figure supplement 2). A1 KO animals scratched significantly less than WT mice after exposure to either bilirubin or the established agonist FMRF, demonstrating that Mrgpra1 is functional in adult mice (Figure 2L–M). The KD of bilirubin towards MRGPRA1 and MRGPRX4 suggests that bilirubin likely does not interact with these receptors in healthy individual