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2 **The calcium-permeable channel OSCA1.3 regulates plant stomatal immunity**

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37 **Summary**

38 Perception of biotic and abiotic stresses often leads to stomatal closure in plants.  
39 Rapid influx of calcium ions ( $\text{Ca}^{2+}$ ) across the plasma membrane plays an important  
40 role in this response, but the identity of  $\text{Ca}^{2+}$  channels involved has remained elusive.  
41 Here, we report that the *Arabidopsis thaliana*  $\text{Ca}^{2+}$ -permeable channel OSCA1.3  
42 controls stomatal closure during immunity. OSCA1.3 is rapidly phosphorylated upon  
43 perception of pathogen-associated molecular patterns (PAMPs). Biochemical and  
44 quantitative phospho-proteomics analyses reveal that the immune receptor-associated  
45 cytosolic kinase BIK1 interacts with and phosphorylates the N-terminal cytosolic loop  
46 of OSCA1.3 within minutes of treatment with the peptidic PAMP flg22 derived from  
47 bacterial flagellin. Genetic and electrophysiological data reveal that OSCA1.3 is  
48 permeable to  $\text{Ca}^{2+}$ , and that BIK1-mediated phosphorylation on its N-terminus  
49 increases this channel activity. Importantly, OSCA1.3 and its phosphorylation by BIK1  
50 are critical for stomatal closure during immunity. Notably, OSCA1.3 does not regulate  
51 stomatal closure upon perception of abscisic acid – a plant hormone associated with  
52 abiotic stresses. Our study thus identifies a long sought-after plant  $\text{Ca}^{2+}$  channel and  
53 its activation mechanisms underlying stomatal closure during immune signaling, and  
54 suggests specificity in  $\text{Ca}^{2+}$  influx mechanisms in response to different stresses.

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58 **Main text**

59 Diverse environmental stimuli induce rapid increases in cytosolic  $\text{Ca}^{2+}$  concentrations  
60 ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) to activate signaling<sup>1</sup>. In plants, rapid and transient  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases are for  
61 example, triggered upon perception of pathogen-associated molecular patterns (PAMPs), or  
62 abiotic stresses, such as hyper-osmolarity, drought or high ozone exposure<sup>2,3</sup>. Leaf stomata,  
63 composed of two guard-cells, mediate water and gas exchanges and show dynamic  $\text{Ca}^{2+}$   
64 responses to such stimuli. Stomata provide natural entry points for plant pathogens<sup>4</sup>, and thus  
65 their closure must be tightly controlled to ensure optimal photosynthesis, while appropriately  
66 restricting evaporation and pathogen entry<sup>5</sup>. Despite the central role of  $[\text{Ca}^{2+}]_{\text{cyt}}$  for stomatal  
67 closure in response to multiple stimuli<sup>6,7</sup>, the identity of the corresponding  $\text{Ca}^{2+}$  channel(s) is  
68 still unknown.

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70 In the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*), the plasma membrane-  
71 associated cytosolic kinase BIK1 and related PBL proteins act as central immune regulators  
72 acting downstream of multiple cell surface immune receptors. BIK1 orchestrates multiple

73 immune outputs triggered upon perception of PAMPs or damage-associated molecular  
74 patterns (DAMPs)<sup>8,9</sup>. Previous work revealed that BIK1 directly phosphorylates the NADPH  
75 oxidase RBOHD to activate ROS production in response to PAMP/DAMP perception<sup>10,11</sup>.  
76 Notably, BIK1 was previously shown to be genetically involved in PAMP-induced Ca<sup>2+</sup> influx  
77 and stomatal closure<sup>11-14</sup>

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79 We therefore hypothesized that BIK1 may directly phosphorylate the elusive Ca<sup>2+</sup> channel(s)  
80 involved in stomatal immunity. Interestingly, Arabidopsis OSCA1.3 (At1g11960), a yet  
81 uncharacterized isoform of the recently described OSCA/TMEM63 family of conserved Ca<sup>2+</sup>  
82 channels<sup>15-19</sup>, is rapidly phosphorylated upon PAMP treatment<sup>20</sup>. Notably, two  
83 phosphopeptides in the predicted first cytoplasmic loop of OSCA1.3 contain a phosphorylated  
84 serine (S) within a motif (SxxL; where x is any aminoacid and L is leucine) conserved in  
85 RBOHD<sup>10,11</sup> (Extended Data Fig. 1). Arabidopsis OSCA1.3 fused to green fluorescent protein  
86 (GFP) localizes to the plasma membrane (Extended Data Fig. 2), consistent with a possible  
87 role in mediating Ca<sup>2+</sup> influx downstream of cell surface immune receptors.

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89 Next, we tested whether OSCA1.3 is a BIK1 substrate. Transiently expressed BIK1 fused to  
90 hemagglutinin (BIK1-HA) co-immunoprecipitated with OSCA1.3-GFP but not GFP-LTI6b, a  
91 plasma membrane marker (Fig. 1a). Treatment with the PAMP flg22 – the ligand of the  
92 immune receptor FLS2 that activates BIK1<sup>21-23</sup> – did not alter association between OSCA1.3-  
93 GFP and BIK1-HA (Fig. 1a). BIK1-HA and OSCA1.3-GFP associations were confirmed in  
94 transgenic Arabidopsis lines, but here flg22 treatment reduced this association (Fig. 1b),  
95 similar to what has been previously observed for BIK1-RBOHD association<sup>10,11</sup>.

96

97 We next sought to determine whether BIK1 phosphorylates OSCA1.3. The previously  
98 described OSCA1.3 phosphosites<sup>20</sup> are within its first cytoplasmic loop (loop1; Extended Data  
99 Fig. 1). *In vitro* pull-down and radioactive kinase assays showed that OSCA1.3-loop1 directly  
100 interacts with and can be phosphorylated by glutathione-S-transferase (GST)-BIK1 (Fig. 2a,b).  
101 This phosphorylation depended on BIK1 kinase activity, since a kinase-dead variant of GST-  
102 BIK1 (GST-BIK1-KD) did not phosphorylate MBP-OSCA1.3-loop1 (Fig. 2b). Targeted  
103 mutagenesis of the identified phosphosites (S49 and S54) and adjacent S50 within OSCA1.3-  
104 loop1 (Extended Data Fig. 1) followed by *in vitro* radioactive kinase assays showed that S54  
105 is the predominant residue phosphorylated by BIK1 (Fig. 2b). Consistent with its partially-  
106 overlapping role with BIK1<sup>10-13,22</sup>, the phylogenetically-related PBL1 kinase could also  
107 specifically phosphorylate OSCA1.3-loop1 at S54 (Extended Data Fig. 3). Notably, flg22-  
108 induced BIK1-dependent phosphorylation on S54 was confirmed *in vivo* by selected-reaction

109 monitoring (SRM) assays (Fig. 2c), further demonstrating that OSCA1.3 is a BIK1 substrate  
110 during immune signaling.

111

112 Arabidopsis has 15 OSCA isoforms grouped in 4 different phylogenetic clades<sup>15,24</sup>. Of these  
113 only OSCA1.1 and OSCA1.2/CSC1 are functionally characterized *in planta* so far, and are  
114 involved in response to osmotic stress<sup>15,16</sup>. Other OSCA isoforms in Arabidopsis and rice  
115 (*Oryza sativa*) have been recently shown to be mechanosensitive non-selective cation  
116 channels proposed in some cases to be Ca<sup>2+</sup>-permeable<sup>24-28</sup>. To test if OSCA1.3 is a Ca<sup>2+</sup>-  
117 permeable channel, we first made use of the Ca<sup>2+</sup>-uptake deficient yeast mutant *cch1/mid1*<sup>29</sup>.  
118 This mutant failed to grow in a halo around a filter paper disc soaked in mating pheromone  $\alpha$   
119 factor, compared to wild-type yeast or the *cch1/mid1* mutant expressing OSCA1.3 (Fig. 3a),  
120 suggesting that OSCA1.3 facilitates Ca<sup>2+</sup> transport in this heterologous system. Expression of  
121 myc-tagged OSCA1.3 in human embryonic kidney 293T (HEK293T) cells and measurements  
122 using the Ca<sup>2+</sup>-sensitive ratiometric fluorescent dye Fura-2 further indicated that OSCA1.3 can  
123 lead to [Ca<sup>2+</sup>]<sub>cyt</sub> increase (Extended Data Fig. 4). Finally, patch-clamp recordings with COS-7  
124 cells revealed currents upon expression of OSCA1.3, which were increased upon BIK1 co-  
125 expression in a kinase activity-dependent and OSCA1.3-S54 phosphorylation-dependent  
126 manner (Fig. 3b,c; Extended Data Figure 5a). Together, these results show that OSCA1.3 is  
127 a BIK1-activated Ca<sup>2+</sup>-permeable channel.

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129 Among OSCA clade 1, only OSCA1.7 (At4g02900) has a motif similar to that of OSCA1.3 at  
130 the same position (Extended Data Fig. 1b). Consistently, OSCA1.7-mediated currents in COS-  
131 7 cells are activated by active BIK1 (Extended Data Fig. 5b,c). Notably, OSCA1.3 and  
132 OSCA1.7 alone were permeable to Ca<sup>2+</sup> and this activity was not increased upon co-  
133 expression of both channels (Fig. 3b,c; Extended Data Fig. 5b,c). We generated a double  
134 homozygous insertional *osca1.3/1.7* null mutant (Extended Data Fig. 6a,b; Extended Data Fig.  
135 7). The overall elevation of [Ca<sup>2+</sup>]<sub>cyt</sub> in response to flg22 treatment in leaf discs of transgenic  
136 wild-type (Col-0) or *osca1.3/1.7* lines expressing the cytosolic Ca<sup>2+</sup> sensor aequorin<sup>12,30</sup> was  
137 comparable (Extended Data Fig. 8a). As OSCA1.3 is preferentially expressed in guard cells  
138 (Extended Data Fig. 7), and BIK1 controls several aspects of stomatal immunity<sup>10,11,22</sup>, we  
139 generated transgenic lines in wild-type (Col-0) or *osca1.3/1.7* backgrounds expressing the  
140 cytosolic ratiometric Ca<sup>2+</sup> sensor YC3.6, which allows measurement of flg22-induced Ca<sup>2+</sup>  
141 spiking with cellular resolution<sup>31</sup>. Single-cell measurement of Ca<sup>2+</sup> spiking in guard cells  
142 showed that the rapid (5 min) flg22-induced Ca<sup>2+</sup> increase was reduced in *osca1.3/1.7*  
143 compared to Col-0 (Fig. 4a; Extended Data Fig. 9a). A similar reduction was observed using  
144 non-invasive microelectrode ion flux measurements (Extended Data Fig. 9b,c). Consistent  
145 with data from aequorin reporter line (Extended Data Fig. 8a), no such decrease was observed

146 in leaf discs of the *osca1.3/1.7* YC3.6 line (Extended Data Fig. 8b), suggesting that the  
147 *osca1.3/1.7* defects are guard cell-specific.

148 Surprisingly, we observed that the quantitatively dampened increase of flg22-induced  $[Ca^{2+}]_{cyt}$   
149 in guard cells correlated with an abolishment of flg22-induced stomatal closure in *osca1.3/1.7*  
150 (Fig. 4b). Notably, stomatal closure in *osca1.3/1.7* was similarly impaired upon treatment with  
151 the DAMP AtPep1 (Fig. 4c). Importantly, stomatal closure in response to the plant stress  
152 hormone abscisic acid (ABA) was however not affected in *osca1.3/1.7* (Fig. 4c), which was  
153 corroborated with stomatal conductance measurements in intact leaves (Fig. 4d, Extended  
154 Data Figure 10). These data reveal that loss of OSCA1.3/1.7 does not generally affect guard  
155 cell physiology, suggesting that OSCA1.3/1.7 play a specific role in stomatal closure during  
156 immunity. Consistently, *osca1.3/1.7* plants were more susceptible than wild-type (Col-0) to the  
157 hypovirulent *Pseudomonas syringae* pv tomato DC3000 *COR* strain to a level comparable to  
158 the immune-deficient mutant *bak1-5* (Fig. 4e).

159 Finally, to test if the role of OSCA1.3/1.7 depends on BIK1-mediated phosphorylation, we  
160 complemented *osca1.3/1.7* with either *OSCA1.3* or *OSCA1.3-S54A*. Expression of *OSCA1.3*,  
161 but not *OSCA1.3-S54A* restored flg22-induced stomatal closure (Fig. 4f). Altogether, our data  
162 demonstrate that *OSCA1.3* is a  $Ca^{2+}$ -permeable channel required for stomatal immunity, the  
163 activation and function of which depend on BIK1-mediated phosphorylation.

164  
165 It is striking that the quantitative reduction of  $Ca^{2+}$  influx observed in single guard cells leads  
166 to a complete abolishment of elicitor-induced stomatal closure. As such, our work identifies a  
167 long-sought after  $Ca^{2+}$  channel involved in early immune signaling, indicative of a threshold  
168 mechanism for the regulation of this important adaptive stress response. We cannot however  
169 completely exclude that *OSCA1.3/1.7* might be permeable to additional cations that may also  
170 contribute to stomatal closure, as other OSCAs have been shown to be non-selective cation  
171 channels<sup>24-28</sup>. Notably, neither *OSCA1.3/1.7* nor their regulation by BIK1 appear to be required  
172 for ABA-induced stomatal closure. These results further support that PAMPs and ABA  
173 distinctly activate components leading to stomatal closure<sup>32,33</sup>. Moreover, our study reveals a  
174 critical activation mechanism for this channel via phosphorylation by BIK1. Several plant  
175 OSCAs have recently been shown to be mechanosensitive  $Ca^{2+}$  channels<sup>24-28</sup>. It remains to  
176 be tested whether *OSCA1.3/1.7* are similarly mechanosensitive, but our results suggest that  
177 phosphorylation by plasma membrane-associated kinases could represent an additional layer  
178 of regulation for this conserved family of  $Ca^{2+}$  channels in response to distinct stimuli, as  
179 recently shown for cyclic nucleotide-gated channels in the context of mesophyll immunity<sup>14,34</sup>.  
180 In the context of immunity, future work is now needed to understand how BIK1 and OSCAs –  
181 together with additional isoforms from other  $Ca^{2+}$  channel families proposed to be involved in  
182 immunity<sup>14,34-38</sup> – help integrate calcium signaling at the plant tissue and organ scales.

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289 **Figure legends**

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291 **Figure 1 | OSCA1.3 associates with BIK1.**

292 **a** Co-immunoprecipitation of BIK1-HA and OSCA1.3-GFP transiently expressed in *N.*  
293 *benthamiana* leaves treated with or without 1  $\mu$ M flg22 for 10 min. GFP-LTI6b served as  
294 negative control.

295 **b** Co-immunoprecipitation of BIK1-HA and OSCA1.3-GFP from *A. thaliana* lines stably  
296 expressing BIK1-HA and OSCA1.3-GFP or GFP-LTI6b, respectively.

297 Immunoprecipitation was performed with  $\alpha$ -GFP agarose beads. Western blots were probed  
298 with  $\alpha$ -GFP and  $\alpha$ -HA. CBB: Coomassie brilliant blue. For blot source data, see  
299 Supplementary Fig.1. Both experiments were performed three times with similar results.

300

301 **Figure 2 | OSCA1.3 is phosphorylated by BIK1 and S54 is a major phosphorylation site.**

302 **a** *In vitro* GST-pulldown with recombinant GST-BIK1 and MBP-OSCA1.3 (aa 30-95). MBP  
303 was used as control. GST-pulldown was performed with glutathione resin and western blots  
304 probed with  $\alpha$ -GST and  $\alpha$ -MBP. For blot source data, see Supplementary Fig.1. The  
305 experiment was repeated three times with similar results.

306 **b** *In vitro* radioactive kinase assay performed with the corresponding recombinant proteins.  
307 For blot source data, see Supplementary Fig.1. The experiment was performed three times  
308 with similar results.

309 **c** SRM relative quantification of tryptic phosphorylated peptide SSPLHS[+80]GALVSK at 0  
310 and 5 min after flg22 treatment. Values are individual points and mean  $\pm$  SE ( $n = 6$ ). \*\*\* $P <$   
311 0.0001 (ordinary one-way ANOVA with multiple comparisons).

312

313 **Figure 3 | OSCA1.3 is a BIK1-activated calcium-permeable channel.**

314 **a** OSCA1.3 complements growth of the calcium-uptake deficient yeast mutant *cch1/mid1*.  
315 Filter discs containing 10  $\mu$ g of the mating pheromone  $\alpha$  factor were placed on nascent lawns  
316 of WT, *cch1/mid1*, or *cch1/mid1* complemented with AtOSCA1.3. DsRed served as control.  
317 Photographs taken after 48 h. OSCA1.3: pYES-DEST52-OSCA1.3, DsRed: pYES-DEST52-  
318 DsRed. The experiment was repeated three times with similar results.

319 **b** Typical currents recorded in whole cell configuration of COS-7 cells expressing OSCA1.3 or  
320 OSCA1.3<sup>S54A</sup> with or without the kinase BIK1 or the mutant BIK1-KD (BIK1<sup>K105A/K106A</sup>). Voltage  
321 pulses were applied from -100 to +60 mV (1.5 s long, 20 mV steps).

322 **c** Current-voltage ( $I/V$ ) curves of currents shown in *b* as indicated on the figure legend ( $n > 3 \pm$   
323 SE). Solutions had two only main charge carriers: Na<sup>+</sup> and Ca<sup>2+</sup>, with equilibrium potentials of  
324 -66.6 mV (Na<sup>+</sup>) and  $> +60$  mV (Ca<sup>2+</sup>) respectively. OSCA1.3 mediated currents crossed the x-

325 line between -10 mV and -20 mV, compatible with the activity of a non-selective cationic  
326 channel permeable to  $\text{Ca}^{2+}$ . Currents recorded at -100 mV in cells expressing OSCA1.3 plus  
327 BIK1 were significantly higher than in cells expressing OSCA1.3 alone (ANOVA6  $p < 0.005$ ).

328

329 **Figure 4 | OSCA1.3 and OSCA1.7 are required for stomatal immunity.**

330 **a** Box and scatterplot showing summed area under the curve (AUC) for wavelet reconstructed  
331 profiles of the first 5 min of flg22-induced calcium spiking in Col-0/YC and *osca1.3/1.7*YC  
332 guard cells. Each point represents the summed AUC for a single cell. Marker shapes represent  
333 different independent experimental repeats and the box plot represents the distribution of all  
334 points for Col-0 or *osca1.3/1.7*. \* $P = 0.0024$  ( $n=4$ , linear mixed effect model plus ANOVA).

335 **b** Stomatal aperture of wild type, *osca1.3*, *osca1.7* and *osca1.3/1.7* plants treated with either  
336 5  $\mu\text{M}$  flg22 or water. Shown are individual data points and mean  $\pm$  SD for  $n > 346$  stomata from  
337 three experiments. \*\*\* $P < 0.0001$  (ordinary one-way ANOVA with multiple comparisons).

338 **c** Stomatal aperture of wild-type and *osca1.3/1.7* plants treated with either water, 5  $\mu\text{M}$  AtPep1  
339 or 10  $\mu\text{M}$  ABA. Shown are individual data points and mean  $\pm$  SD for  $n > 410$  stomata from three  
340 experiments. \*\*\* $P < 0.0001$  (ordinary one-way ANOVA with multiple comparisons).

341 **d** Leaf transpiration recorded in excised intact leaves of wild-type and *osca1.3/1.7* plants.  
342 Stimuli were added to the solution at the petioles to concentrations of 10  $\mu\text{M}$  flg22, 10  $\mu\text{M}$  ABA  
343 or 0.01 % ethanol as control. Data show mean  $\pm$  SEM for  $n=4-5$  leaves. The experiment was  
344 performed twice with similar results.

345 **e** Numbers of *Pto* DC3000 *COR* bacteria determined 3 days after spray inoculation in Col-0,  
346 *osca1.3/1.7* and *bak1-5* plants. Shown are individual data points and mean  $\pm$  SD for  $n=22$  to  
347 24 plants from three experiments. \* $P = 0.012$  (ordinary one-way ANOVA with multiple  
348 comparisons).

349 **f** Stomatal aperture of wild-type, *osca1.3/1.7* and *osca1.3/1.7* complemented with  
350 *pOSCA1.3:OSCA1.3(WT)* or *pOSCA1.3:OSCA1.3(S54A)* plants treated with either 5  $\mu\text{M}$  flg22  
351 or water. Shown are individual data points and mean  $\pm$  SD for  $n > 108$ . \*\*\* $P < 0.0001$  (ordinary  
352 one-way ANOVA with multiple comparisons). The experiment was repeated three times with  
353 similar results.

354

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359 **Methods**

360 No statistical methods were used to predetermine sample size. The experiments were not  
361 randomized and investigators were not blinded to allocation during experiments and outcome  
362 assessment.

363

### 364 **Plant material and growth conditions**

365 All *Arabidopsis thaliana* lines used in this study were in the Col-0 ecotype background. Lines  
366 *osca1.3* (SALK\_134381) and *osca1.7* (SALK\_114694) were obtained from the Nottingham  
367 Arabidopsis Stock Centre (NASC) and genotyped for homozygosity using left border and  
368 gene-specific primers listed in Extended Data Table 2. Line *osca1.3/1.7* was obtained by  
369 crossing *osca1.3* and *osca1.7* and screening the F2 for double homozygous progeny. *bak1-5*  
370 has been described previously<sup>39</sup>. Unless stated otherwise, plants were grown on soil as one  
371 plant per pot with a 10-h photoperiod at 20 to 22 °C in environmentally controlled growth  
372 rooms. Four-to-five-week-old plants were used for experiments unless stated otherwise. Col-  
373 0 plants stably expressing Yellow Cameleon 3.6 under the *ubiquitin10* promoter were kindly  
374 provided by Myriam Charpentier. Mutant plants were crossed with this line and progeny  
375 screened for homozygosity of the T-DNA insertions and the presence of the YC3.6 reporter.  
376 Lines expressing the calcium reporter aequorin under the control of the 35S promoter were  
377 generated by transforming Col-0, *osca1.3*, *osca1.7* and *osca1.3/1.7* with the construct  
378 pB7WG2:aequorin via agrobacterium-mediated transformation. Selection of transformants  
379 was performed on BASTA-containing full strength MS medium and transformants were  
380 screened for similar aequorin levels in the T1 generation via western blot with  $\alpha$ -aequorin  
381 antibody (Abcam ab9096). T2 plants were used for assays. Complementation lines were  
382 generated by transforming *osca1.3/1.7* plants with pGWB1-pOSCA1.3:OSCA1.3(WT) or  
383 pGWB1-pOSCA1.3:OSCA1.3(S54A) by agrobacterium-mediated transformation. T1 plants  
384 were selected on hygromycin-containing MS medium supplemented with 1 % sucrose and  
385 directly used for stomatal aperture assays. Col-0 and *osca1.3/1.7* plants were grown in parallel  
386 under the same conditions on non-selective medium. Expression levels for OSCA1.3 were  
387 checked via qRT-PCR to document complementation (Extended Data Fig. 6c). Double  
388 transgenic lines were generated by crossing *pBIK:BIK1-HA* line<sup>10,22</sup> with *p35S:GFP-LT16b*  
389 line<sup>40</sup> or transforming *pBIK:BIK1-HA* plants with construct *p35S:OSCA1.3-GFP* via  
390 Agrobacterium-mediated transformation.

391

### 392 **Chemicals**

393 Synthetic flg22, elf18 and AtPep1 were purchased from EZBiolab and dissolved in sterile  
394 water. ABA was purchased from Sigma-Aldrich.

395

### 396 **Homology modeling for OSCA1.3**

397 SWISS-MODEL<sup>41</sup> and HHPRED<sup>42</sup> were used to search for structural homologs to full length  
398 OSCA1.3. The structural modeling of OSCA1.3 was performed using SWISS-MODEL<sup>41</sup> with  
399 OSCA1.2 (PDB-ID: 6MGV; ref. 26) as template. Images were created with CHIMERA<sup>43</sup>.

400

#### 401 **Molecular cloning**

402 For OSCA1.3 subcellular localization detection in *Arabidopsis*, the fragment of the promoter  
403 region (1226 bp) and the coding region of OSCA1.3 genomic DNA was amplified and inserted  
404 into Entry vector pCR<sup>TM</sup>8 (Invitrogen<sup>TM</sup>) via TOPO-TA cloning , and then introduced into  
405 Gateway binary vector pGWB4 with a GFP tag at the C-terminus after recombination by LR  
406 Clonase II (Invitrogen). For protein expression in *N. benthamiana*, we generated epiGreenB-  
407 p35S:OSCA1.3-GFP by inserting OSCA1.3 cDNA fragment into epiGreenB (eGFP) vector  
408 using In-fusion enzyme (Clontech Laboratories), and utilized previous reported pGWB14-  
409 p35S:BIK1-3xHA<sup>44</sup> as well as p35S:GFP-LTI6b<sup>40</sup> constructs. Site-directed mutagenesis of  
410 OSCA1.3 was achieved by PCR using overlapping primers containing the desired point  
411 mutations. To generate constructs for Arabidopsis complementation assay,  
412 *pOSCA1.3:OSCA1.3(WT)* and *pOSCA1.3:OSCA1.3(S54A)* were cloned into Entry vector  
413 pCR<sup>TM</sup>8 and then introduced into gateway binary vector pGWB1 with no epitope tag<sup>45</sup>. For  
414 protein expression in *E. coli*, OSCA1.3 (88- 285 bp) and its mutation variants were cloned into  
415 pOPINM vector using in-fusion enzyme to generate N-terminal 6xHIS-MBP fusion. GST-BIK1  
416 and GST-BIK1-KD (kinase dead) constructs were described previously<sup>23</sup>. GST-PBL1 and  
417 GST-PBL1-KD fusions were created after recombination using respective entry clones and  
418 gateway vector pABD72\_pGEX-2TMGW. For expression in COS-7 cells, coding sequences  
419 of OSCA1.3, OSCA1.3<sup>S54A</sup>, BIK1 and BIK1-KD (BIK1<sup>K105A/K106A</sup>, ref. 44) were PCR-amplified  
420 with primers listed in Table S2 and cloned into the vector pCI (Promega) via restriction enzyme  
421 cloning. The coding sequence of OSCA1.7 was synthesized with the corresponding restriction  
422 sites and subcloned into pCI. For expression in yeast, the OSCA1.3 coding sequence was  
423 converted to yeast codon usage using Geneious® 8.1.8, synthesised by Life Technologies<sup>TM</sup>  
424 (ThermoFisher Scientific) into the entry vector pENTR221 and subsequently cloned into the  
425 destination vector pYES-DEST52 with Gateway<sup>TM</sup> LR Clonase<sup>TM</sup> II Enzyme Mix  
426 (Invitrogen<sup>TM</sup>).

427

#### 428 **Protein expression and purification**

429 For protein purification, constructs were transformed into the *E. coli* expression strain BL21  
430 (DE3). The bacterial culture was grown to an OD<sub>600</sub> of 0.6, and 0.5 mM IPTG was then added  
431 to induce protein expression. The induction continued at 16 °C overnight. HIS-MBP-OSCA1.3  
432 variants were purified using nickel resin with buffer A (50 mM Tris-HCl, pH 8.0, 500 mM NaCl,  
433 5 % Glycerol, and 20 mM imidazole) containing 0.5 mM DTT and 0.2 mM PMSF as lysis buffer.

434 Purified proteins were eluted in buffer B (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 % Glycerol,  
435 and 200 mM imidazole) after 5 washes using buffer A. GST-BIK1/PBL1 was purified using  
436 glutathione resin. Buffer C (20 mM Tris-HCl, pH 7.5, and 500 mM NaCl) with 0.5 mM DTT and  
437 0.2 mM PMSF was used as lysis buffer and buffer D (20 mM Tris-HCl, 500 mM NaCl, and 20  
438 mM reduced glutathione, pH adjusted to 7.0) was used as elution buffer. After purification, all  
439 proteins were dialysed into buffer E (20 mM Tris-HCl, pH7.5, 150 mM NaCl, and 5 mM DTT)  
440 for further application.

441

#### 442 **Co-immunoprecipitation in *N. benthamiana***

443 Two leaves of 4- to 5-week-old *N. benthamiana* plants were syringe-infiltrated with  
444 *Agrobacterium* strain GV3101 expressing GFP-OSCA1.3 and BIK1-HA. Two days later,  
445 leaves were cut and halves treated with either 1  $\mu$ M flg22 or mock for 10 min. The tissue was  
446 ground in liquid nitrogen and homogenized in extraction buffer (0.5 % (w/v) PVPP, 150 mM  
447 Tris-HCl, pH 7.5, 150 mM NaCl, 10 % glycerol, 10 mM EDTA, 1 mM NaF, 1mM NaMo, 1.5  
448 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM DTT, 1 % protease inhibitor cocktail (Sigma Aldrich), and 1 mM PMSF)  
449 with 1% IGEPAL CA-630. The supernatant obtained after centrifugation was incubated with  
450 25  $\mu$ L of GFP-Trap<sup>®</sup> agarose beads (ChromoTek). Following an incubation for several hours  
451 at 4 °C, the beads were washed 3 times using extraction buffer with 0.5 % IGEPAL CA-630  
452 before SDS-PAGE and Western blot detection with  $\alpha$ -GFP and  $\alpha$ -HA (Santa Cruz). For blot  
453 source data, see Supplementary Fig.1.

454

#### 455 **Co-immunoprecipitation in Arabidopsis**

456 Sterilized seeds were sown on MS agar plates. After stratification for 3 days in the dark at 4  
457 °C, seeds were transferred to light. Four days later, ten seedlings were transferred into each  
458 well of a 6-well plate containing liquid MS. Two-week-old seedlings from two 6-well plates  
459 were elicited by 1  $\mu$ M flg22 for 10 min. MS medium treatment was used as a control. Tissue  
460 was ground in liquid nitrogen and extraction buffer (150 mM Tris-HCl, pH 7.5, 150 mM NaCl,  
461 10 % glycerol, 5 mM EDTA, 10 mM NaF, 10 mM NaMo, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM DTT, 1x protease  
462 inhibitor cocktail 1, 1x protein phosphatase inhibitor cocktail 2 (Sigma Aldrich), and 1 mM  
463 PMSF) containing 2 % IGEPAL CA-630 was added to the resulting powder at 2 mL/g tissue.  
464 After homogenizing for 1 h, samples were centrifuged for 20 min at 13,000 rpm at 4 °C. The  
465 concentration of IGEPAL CA-630 in the supernatant was adjusted to 0.5 % by diluting the  
466 samples with extraction buffer. For immunoprecipitation, 100  $\mu$ L of  $\alpha$ -GFP agarose beads  
467 (Chromotek) were added. After incubation for 2 h, beads were washed 3 times using extraction  
468 buffer containing 0.5% IGEPAL CA-630 before SDS-PAGE and western-blot detection with  $\alpha$ -  
469 GFP and  $\alpha$ -HA (Santa Cruz). For gel and blot source data, see Supplementary Fig.1.

470

#### 471 ***In vitro* GST pull-down**

472 Glutathione resin Sepharose 4 Fast Flow (GE Healthcare) was equilibrated with incubation  
473 buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 % Tween 20, 1 mM DTT, and 100  
474 μM PMSF). Ten micrograms of the GST fusion proteins were incubated with the resin in  
475 incubation buffer for 2 h. Subsequently, the resin was washed 3 times with incubation buffer  
476 before the second incubation with 10 μg of MBP fusion proteins. After 1 h incubation, the resin  
477 was washed 5 times and boiled in 6x SDS loading buffer for SDS-PAGE and western blot  
478 detection with α-GST (Santa Cruz) and α-MBP (New England Biolabs). For blot source data,  
479 see Supplementary Fig.1.

480

#### 481 ***In vitro* kinase assay**

482 One microgram of both kinase as well as substrate were mixed up to 20 μL in buffer containing  
483 50 mM Tris-HCl, pH 7.5 and 3 mM MnCl<sub>2</sub>. Five microliters of 5x kinase buffer (25 mM MnCl<sub>2</sub>,  
484 5 mM DTT and 5 μM unlabelled ATP) was added to each reaction. Every reaction was  
485 incubated with 183 KBq of [<sup>32</sup>P]-γ-ATP for 30 min at 30 °C while shaking. Reactions were  
486 stopped by adding 6x SDS loading buffer. After SDS-PAGE separation, proteins were  
487 transferred onto PVDF membranes followed by staining with CBB. Phosphorylation of proteins  
488 was detected by autoradiography using a FUJI Film FLA5000 PhosphorImager (Fuji, Tokyo,  
489 Japan). For blot source data, see Supplementary Fig.1.

490

#### 491 **Confocal laser scanning microscopy (CLSM)**

492 Cotyledons of Arabidopsis seedlings were imaged on a Leica TCS SP5 (Leica, Germany)  
493 confocal microscope using a 63 × 1.2 NA water immersion objective. GFP was excited using  
494 the Argon ion laser line 488 nm. Fluorescence emission was collected within following band  
495 width generated by an AOTF: 500–540 nm for GFP. Confocal micrographs were analysed and  
496 modified using FIJI (ImageJ 2.0.0–39/rc-1.50b).

497

#### 498 **Seedling growth and elicitation with flg22 (for SRM)**

499 Approximately 20 mg of sterilised seeds were sown into a 250 mL sterile conical flask  
500 containing 50 mL liquid medium (1/2 MS salts, 1 % (w/v) sucrose, pH 5.7), sealed with foil  
501 wrapping and chilled for 48 h, 4 °C in darkness. Flasks were transferred to an orbital shaker  
502 (New Brunswick™ Innova® 2300) rotating at 140 rpm in a 16 h light/8 h dark photoperiod at  
503 21 °C. After 7 d, the seedling clumps were vacuum infiltrated with 1 μM flg22 peptide for 1  
504 min with shaking before releasing to atmospheric pressure. Excess liquid was removed from

505 the clumps and clumps were frozen in liquid nitrogen after 5 min exposure to flg22. Untreated  
506 ( $t_0$ ) controls were only vacuum infiltrated before drying and freezing.

507

#### 508 **Protein extraction and trypsin digestion (for SRM)**

509 Frozen seedling clumps were ground to a coarse powder in liquid nitrogen and further  
510 disrupted using a Braun 853202 homogenizer (B. Braun Melsungen AG) at 1200 rpm for 5  
511 min with a Potter-Elvehjem glass pestle in a 30 mL glass tube (Sartorius) containing 10 mL  
512 ice-cold kinase extraction buffer [50 mM Tris pH7.5, 10 % glycerol, 2 mM DTT, 10 mM NaF,  
513 10 mM  $\text{Na}_2\text{VO}_4$ , 5 mM EDTA, 50 mM  $\beta$ -glycero-phosphate, 1 mM PMSF and 100  $\mu\text{L}$  protease  
514 inhibitor cocktail (SIGMA)] surrounded with an ice jacket. Crude extracts were centrifuged at  
515 4,300  $g$ , 1 h, 4  $^\circ\text{C}$  to remove cell debris followed by ultracentrifugation at 100,000  $g$ , 30 min, 4  
516  $^\circ\text{C}$  to create a microsome-enriched pellet. After removal of supernatant the pellet was  
517 solubilized in 8 M urea/50 mM ammonium bicarbonate to denature proteins.

518 Up to 3 mg of protein was reduced with 5 mM tris(2-carboxyethyl)phosphine 20 min, 37  $^\circ\text{C}$ ,  
519 200 rpm then alkylated with 40 mM iodoacetamide, during 60 min at 25  $^\circ\text{C}$ , under shaking at  
520 200 rpm. Samples were diluted in 5 volumes 50 mM ammonium bicarbonate to reduce urea  
521 concentration. Sequencing grade trypsin (Thermo) was added at 1:100 (w/w)  
522 enzyme:substrate and incubated for 16 h, 37  $^\circ\text{C}$ , 200 rpm. The reaction was stopped by  
523 acidification with 1% (v/v) trifluoroacetic acid. Peptides were cleaned-up using C18 silica  
524 reversed-phase chromatography columns (Sep-Pak) according to the manufacturer's  
525 instructions and the final eluates dehydrated in an acid resistant speed-vac.

526

#### 527 **Phospho-peptide enrichment (for SRM)**

528 Lyophilized tryptic peptides were resuspended by sonication in phthalic acid/80% acetonitrile  
529 (0.1 g/mL) solution which had been further acidified with 3.6% (v/v) trifluoroacetic acid. The  
530 peptide solution was loaded into a Mobicol spin column containing 1.56 mg  $\text{TiO}_2$ -coated  
531 particles (Titanosphere) that had been previously washed in MeOH and equilibrated in phthalic  
532 acid/acetonitrile solution (above). The sealed columns containing the peptide/ $\text{TiO}_2$  solution  
533 were incubated for 45 min on a head-over-tail rotor followed by washes in phthalic  
534 acid/acetonitrile solution, 80 % (v/v) acetonitrile/0.1% trifluoroacetic acid, 0.1 % (v/v)  
535 trifluoroacetic acid. Peptides were eluted with  $\text{NH}_4\text{OH}$  solution (pH 10.5) into a sufficient  
536 amount (usually 60-80  $\mu\text{L}$  of 10 % (v/v) trifluoroacetic acid to give a final pH of 2-3. The  
537 enriched phospho-peptide solution was cleaned using C18 MicroSpin Columns (The Nest  
538 Group Inc) and eluted into low-bind microfuge tubes with 40 % (v/v) acetonitrile.

539

#### 540 **Identification of proteins and phospho-peptides by LC-MS/MS (for SRM)**

541 LC-MS/MS analysis was performed using a Fusion-Orbitrap mass spectrometer (Thermo  
542 Scientific) and a U-3000 nanoflow-HPLC system (Thermo Scientific) as described  
543 previously<sup>46</sup>. The entire TAIR10 database was searched (www.Arabidopsis.org) using Mascot  
544 (v 2.3.02, Matrix Science) (with the inclusion of sequences of common contaminants, such as  
545 keratins and trypsin). Parameters were set for 10 ppm peptide mass tolerance and allowing  
546 for Met oxidation and two missed tryptic cleavages. Carbamidomethylation of Cys residues  
547 was specified as a fixed modification, and oxidation of Met and phosphorylation of Ser, Tyr or  
548 Thr residues were allowed as variable modifications. Scaffold (v3; Proteome Software) was  
549 used to validate MS/MS-based peptide and protein identifications and annotate spectra. The  
550 position and quality of spectra for phospho-peptides were also manually examined before  
551 acceptance.

552

### 553 **SRM analysis and relative quantification of phosphorylation**

554 Synthetic peptides (JPT Peptide Technologies) for OSCA1.3 pSSPLHSGALVSK,  
555 SpSPLHSGALVSK and SSPLHpSGALVSK were used to optimise an SRM method for  
556 detection in the phospho-peptide enriched samples using the program Skyline<sup>47</sup>. Control  
557 peptides used for normalisation were selected from an initial shortlist of 30 based on their  
558 spectral counts in each sample not deviating +/- 25 % from the median value of all samples.  
559 An SRM method was designed to measure these peptides with better resolution but this time  
560 to confirm that the average intensity in each sample did not deviate +/- 1 standard deviation  
561 from the mean intensity of all samples. Retention times and transitions were confirmed by  
562 targeting the control peptides in a 15N-labelled phospho-peptide mix derived from total  
563 Arabidopsis protein. Eight control peptides with a similar dynamic range were selected for  
564 normalisation and incorporated into the SRM method containing the SSPLHSGALVSK  
565 phospho-peptide variants given below (Extended Data Table 1). iRTs (Biognosys) were added  
566 to each injection to track and correct for retention time changes. Peptide sequence, precursor  
567 m/z and transitions are specified in Extended Data Table 1.

568 SRM analysis was performed using nano-spray ESI and a TQ-S MS (Waters Corp., MA, USA).  
569 The LC system consisted of a nanoAcquity with a Symmetry trap (Waters, C18, 180 µm × 20  
570 mm) to concentrate and desalt the peptides before elution to the analytical column (Waters,  
571 CSH 250 mm C18 columns, 75 µm i.d., 1.7 µm beads). A flow rate of 250 nL/min was used  
572 with a gradient from 3% acetonitrile to 65 % acetonitrile over 90 min. One or two injections  
573 were performed from one to three independent biological replicates. The resultant TQ-S files  
574 were imported into Skyline and the peak definitions checked manually. The peak areas were  
575 then exported into Excel (Microsoft) for further analysis. The summed intensity of each  
576 OSCA1.3 phospho-peptide was normalised (by division) against the summed intensities of the  
577 eight control peptides for relative quantification. All SRM assay information and raw data have



578 been deposited to the Panorama Skyline server and can be accessed via:  
579 ([https://panoramaweb.org/labkey/project/Sainsbury Lab-](https://panoramaweb.org/labkey/project/Sainsbury%20Lab-Proteomics/xxxx/xxxxx_SRM/begin.view) TSL  
580 [Proteomics/xxxx/xxxxx\\_SRM/begin.view](https://panoramaweb.org/labkey/project/Sainsbury%20Lab-Proteomics/xxxx/xxxxx_SRM/begin.view))  
581

582

### 583 **Yeast complementation**

584 Yeast complementation was performed as described in ref. 48. In brief, the *cch1/mid1* mutant<sup>49</sup>  
585 was transformed via the lithium acetate method<sup>50</sup> with either the vector pYES-DEST52  
586 (Invitrogen) expressing Ds-Red or pYES-DEST52 expressing OSCA1.3 (codon bias corrected  
587 for yeast expression) and transformants selected on yeast minimal medium without uracil. To  
588 test for complementation, sterile cellulose filter discs (6 mm diameter and 45 µm pore size)  
589 were soaked with 10 µg of synthetic alpha factor (Sigma T6901) and placed on nascent lawns  
590 of WT (JK9-3da (*MATa*, *leu2-3, 112*, *his4*, *trp1*, *ura3-52*, *rme1*)) or the transformed *cch1/mid1*  
591 mutants and pictures taken after 48 h of growth at 30 °C.

592

### 593 **COS-7 cell transfection and patch-clamp**

594 COS-7 cells (provided from ATCC, Manassas, VA, USA) were used at low passage ( $P < 7$ ).  
595 They were maintained at 37 °C and 5 % CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium,  
596 supplemented with 5 % fetal bovine serum and 1 % penicillin/streptomycin (Gibco,  
597 Thermofisher). The coding sequences of OSCA1.3, OSCA1.3-S54A, OSCA1.7, BIK1 and  
598 BIK1-KD were introduced into pCI (Promega, Madison, WI, USA). COS cells were plated at a  
599 density at 50% confluence in 35-mm-diameter dishes and transfected using FugeneHD  
600 (Promega, Madison, WI, USA) as specified by the supplier. Cells were transfected with pCI-  
601 OSCA1.3 (0.4 µg), pCI-OSCA1.3-S54A (0.4 µg), pCI-OSCA1.7 (0.4 µg) or pCI-OSCA1.3 (0.2  
602 µg) plus pCI-OSCA1.7 (0.2 µg), with pCI-BIK1 (0.4 µg), pCI-BIK1-KD (0.4 µg) or pCI (0.4 µg).  
603 PIRE5-CD8 (0.05 µg) was co-transfected to select expressing cells<sup>51</sup>. Cells were transferred  
604 in new petri dishes 36 hours after transfection (by trypsin treatment), at low density for patch-  
605 clamp study. Cells were analyzed 36 to 40 h after transfection. Transfected cells were detected  
606 with the anti-CD8 antibody-coated bead method (Dynabeads CD8, Thermofisher<sup>52</sup>). Pipettes  
607 were pulled with a P97 puller (Sutter Instrument, Novato, CA, USA). Their resistance was: 3-  
608 5 Mohm. Currents were recorded after establishing the whole-cell configuration<sup>53</sup>, filtered at  
609 1-2 kHz with a sampling frequency of 2-4 kHz using an Axopatch 200A amplifier, digidata 1200  
610 series interface and Clamfit6 software (Molecular device, San Jose, CA, USA). Except for Ext.  
611 Data Fig 5a, the pipette solution contained 140 mM Na-Gluconate, 3 mM MgCl<sub>2</sub>, 4 mM HCl, 5  
612 mM EGTA, and 10 mM Bis-tris propane pH 7.2 (Hepes). Except for Ext. Data Fig 5a, the bath  
613 solution contained 10 mM Na-Gluconate, 20 mM Ca-Gluconate, and 10 mM Bis-tris propane,  
614 pH 6.5 (MES). Ext. Data Fig.5a pipette solution: MgCl<sub>2</sub> 3 mM, EGTA 5 mM, HCl 4 mM, Bis-

615 tris propane pH 7.2 (Hepes). Ext. Data Fig.5a bath solution: CaCl<sub>2</sub> 5mM, Bis-tris propane pH  
616 6.5 (MES). Ca-gluconate was added to the standard bath solution to increase external calcium  
617 concentration to 25, 45 and 65 mM successively. The junction potentials of the different  
618 solutions in Ext. Data Fig. 5a were calculated using pClamp6 software and corrected  
619 accordingly. Solutions were adjusted to 350 mosmol.kg<sup>-1</sup> with D-mannitol. Voltage protocol:  
620 1.5 s pulses from -100 to +60 mV (20 mV steps), holding potential 0 mV.

621

### 622 **Calcium measurements in HEK cells**

623 HEK293T cells (ATCC, #CRL-3216) were maintained at 37 °C and 5 % CO<sub>2</sub> in Dulbecco's  
624 Modified Eagle's Medium F12-HAM (Sigma-Aldrich), supplemented with 10% fetal bovine  
625 serum, 15 mM HEPES, and 1% penicillin/streptomycin. For calcium experiments, cells were  
626 seeded on black, clear-bottom, half-volume 96-well plates coated with polyethylenimine (25  
627 µg/mL for 1 h at 37 °C; Sigma-Aldrich). Cells were transiently transfected using GeneJuice  
628 (Novagen) according to the manufacturer's instructions.

629 Calcium measurements were performed 40 h post-transfection. Cells were loaded for 1 h at  
630 37 °C with a 1:1 mixture of Fura-2-QBT calcium kit (Molecular Devices) and calcium-free NaE  
631 buffer (137 mM NaCl, 5 mM KCl, 1.2 mM MgCl<sub>2</sub>, 4.2 mM NaHCO<sub>3</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM  
632 HEPES, adjusted to pH 7.4 with NaOH), plus 10 mM glucose and 2 mM probenecid.  
633 Intracellular Ca<sup>2+</sup> was assessed by measuring changes in fluorescence with a FlexStation 3  
634 fluorescence plate reader (Molecular Devices) at 37 °C. Measurements were recorded at  
635 340/510 nm and 380/510 nm every 6 seconds for a total of 530 s. Additions of sorbitol were  
636 made at 30 s (final concentration 1.3 M) and CaCl<sub>2</sub> at 150 s (final concentration 0.6 mM). Data  
637 were presented as the ratio of the 340/380 measurements and were normalized to the  
638 baseline prior to additions.

639

### 640 **Calcium measurements in aequorin lines**

641 Twelve leaf discs per line from 6 individual plants were incubated in a 12.5 µM coelenterazine  
642 h (Cayman Chemical) solution overnight to reconstitute aequorin. The next day, the  
643 coelenterazine solution was replaced by water and luminescence measured in a Synergy H1  
644 plate reader (BioTek) with a measuring time of 40 ms and a 30-s interval. After 10 min, flg22  
645 was added to a final concentration of 100 nM and measurement was continued for another 45  
646 min before discharging with a calcium chloride/ethanol solution to a final concentration of  
647 1M/10%. Discharging values were measured for 99 s. Background luminescence was  
648 subtracted and cytosolic calcium concentrations were calculated as previously described<sup>54</sup>.

649

### 650 **Calcium measurements in leaf disc of YC3.6 lines**

651 Leaf discs ( $\varnothing$  4mm) of 3- to 5-week-old *A. thaliana* plants were harvested with a biopsy punch  
652 and dark incubated at room temperature overnight in a 96-well plate in 0.1 mL deionized water  
653 with the abaxial site up. Fluorescence measurements were carried out in a Synergy H1 hybrid  
654 plate reader (BioTek Instruments, USA) equipped with a Xenon flash lamp. In 45 s intervals  
655 CFP was excited at 440 nm and emission signals were detected at 480 nm (CFP) and 530 nm  
656 (YFP). Flg22 was added to a final concentration of 1  $\mu$ M through a build-in dispenser system.  
657 For quantification of the signal, YFP emission at CFP excitation was divided by CFP emission  
658 at CFP excitation.

659

### 660 **Calcium measurements in guard cells of YC3.6 lines**

661 Ratiometric calcium measurements in guard cells were performed in epidermal strips as  
662 previously described<sup>31</sup>. Briefly, leaf discs were stuck onto coverglasses using medical  
663 adhesive (Hollister, Libertyville, IL, USA) with the lower epidermis facing the glass. All tissues  
664 except for the epidermis were gently removed using a razor blade. Strips were incubated in  
665 water overnight in a plant growth chamber at 22 °C and in the light for several hours before  
666 starting the measurement. Before the measurement, a chamber was formed around the strip  
667 using Carolina Observation Gel (Carolina Biological Supply Company) and filled with 270  $\mu$ L  
668 of water. The coverslip was taped onto a platform and mounted onto a Nikon Eclipse Ti  
669 inverted microscope. Excitation was performed at a wavelength of 430/24nm using a blue light  
670 LED (LXK2-PB14-Q00, Lumileds) and an ET430/24x excitation filter (Chroma). The  
671 microscope was equipped with a 89002bs dual band-pass dichroic mirror (Chroma). CFP and  
672 YFP emission fluorescence were separated using an optosplit device (Cairn Research) with  
673 a T495LPXR dichroic mirror and an ET470/24m filter for CFP and ET535/30m filter for YFP  
674 (Chroma). Images were captured with a RETIGA-SRV CCD camera (Qimaging). Recording  
675 was performed using Metafluor 7.8.9.0 software (Universal Imaging). Single guard cells were  
676 defined as regions-of-interest. Cells were observed for 5 min at 20-s frame intervals, followed  
677 by 5 min at 5-s intervals, before flg22 was added to the bath at time point 10 min. Cells which  
678 during this 10-min period showed oscillations (so-called spontaneous oscillations) and just  
679 continued to do so after the addition of flg22 were excluded from the analysis as it would not  
680 be possible to state that the oscillations after the addition of flg22 were caused by the flg22 as  
681 they have been observed already before it was added. Flg22 was added from a x10 stock in  
682 MilliQ-H<sub>2</sub>O to yield a final concentration of 1  $\mu$ M. Analysis was performed using Fiji<sup>55</sup>. Ratio  
683 values were determined by dividing YFP by CFP intensities.

684 Oscillations induced by flg22 in guard cells do not show a defined frequency or period, and  
685 different cells, also those belonging to the same stomate, are not synchronized<sup>31</sup>. In addition,  
686 peaks often do not return to the baseline before the launch of a new spike. This is in contrast  
687 to, for example, the very regular Nod factor-induced spiking, where parameters such as

688 period, frequency and number of spikes can easily be determined<sup>56</sup>, or calcium signals  
689 induced by stresses such as osmotic or salt treatment, which are characterized by one defined  
690 fast-occurring peak, which can easily be described by its height<sup>15</sup>. Oscillations induced by  
691 flg22 last for around 30 minutes. Measuring with YC3.6 over this time period results in  
692 bleaching of the reporter over time, whereby YFP and CFP differ in their bleaching  
693 characteristics, *i.e.* YFP is bleaching faster. This results in a ratio baseline, which often is  
694 neither straight nor linear, and therefore the height of a given peak during the measurement –  
695 especially if it is one that has not originated from the baseline – cannot easily be determined.  
696 For the same reason, just determining the sum of all values to integrate the signal would not  
697 be correct. To account for the normally occurring variability in spiking between cells and the  
698 chaotic nature of the oscillations, we analysed the area under the curve in the first five minutes  
699 after flg22-treatment as parameter, which represents the speed and strength of the first influx  
700 of calcium over the plasma membrane in an objective way. For every replicate, the exact time  
701 point of addition of flg22 was set as start time and the analysis performed from the start time  
702 to the start time + 5 min. Wavelet analysis was chosen to account for correct determination of  
703 baseline and peaks. The wavelet analysis produces a wave that is centred around 0 with  
704 positive and negative peaks, removing the need to define a basal line and instead taking the  
705  $y = 0$ . Hence, the AUC can be calculated simply using the trapezoid rule. Original curves and  
706 a description of how this analysis was performed are available as supplementary data  
707 (Supplementary Data 1) and on [https://github.com/TeamMacLean/peak\\_analysis](https://github.com/TeamMacLean/peak_analysis).

708

### 709 **Calcium-flux measurements in guard cells**

710 Guard cell preparation: Net  $\text{Ca}^{2+}$  fluxes were measured non-invasively using SISE (Scanning  
711 Ion Selective Electrodes<sup>57,58</sup>) technique with guard cells in isolated epidermal strips. Lower  
712 epidermis from 5- to 6-week-old leaves via double-sided adhesive tape were mounted to the  
713 recording chamber and incubated in buffer based on 1 mM KCl, 1 mM  $\text{CaCl}_2$ , and 10 mM  
714 MES, pH 6.0 (Bis-tris propane) overnight. Following adaptation to the stomatal opening pre-  
715 stimulus conditions flg22 was added into the bath solution at final concentration of 1  $\mu\text{M}$ .

### 716 Electrode preparation, calibration and experimental set-up for ion flux measurements:

717 The electrodes were pulled from borosilicate glass capillaries w/o filament ( $\varnothing$  1.0 mm, Science  
718 Products GmbH) with a vertical puller (Narishige Scientific Instrument Lab). They were baked  
719 over night at 220°C and silanized with N,N-Dimethyltrimethylsilylamine (Sigma-Aldrich) for 1  
720 h.  $\text{Ca}^{2+}$  selective electrodes were backfilled with 500 mM  $\text{CaCl}_2$  and tip filled with calcium  
721 ionophore I cocktail A (Sigma-Aldrich). Calibration of  $\text{Ca}^{2+}$  selective electrodes was performed  
722 in solutions containing 10, 1 and 0.1 mM  $\text{CaCl}_2$ . For lanthanum experiments, electrodes were  
723 calibrated with a 1 mM lanthanum background. Only electrodes were used that recorded a  
724 shift in voltage of approximately 29 mV per pCa unit. The ion selective electrodes were

725 positioned with a Micromanipulator (PatchStar, Scientifica) at approx. 2  $\mu\text{m}$  distance to a guard  
726 cell using an inverted microscope (Axiovert 135, Carl Zeiss AG). The electrode was connected  
727 via Ag/AgCl half-cells to the head stage of the microelectrode amplifier (custom-built).  
728 Electrode was scanning at 10 s intervals over a distance of 29  $\mu\text{m}$ , using a piezo stepper  
729 (Luigs & Neumann GmbH). Raw data were acquired with a NI USB 6259 interface (National  
730 Instruments), using custom-built Labview-based software "Ion Flux Monitor"<sup>57</sup>. Raw voltage  
731 data were converted offline into ion flux data, as described<sup>57-60</sup>. For reasons of comparability,  
732 all measurements were converted with the same settings in "Ion Flux Monitor". A detailed  
733 description of the statistical analysis performed is available here:  
734 [https://github.com/TeamMacLean/peak\\_analysis](https://github.com/TeamMacLean/peak_analysis).

735

### 736 **Stomatal aperture assays**

737 Leaf discs (two leaf discs per plant, three plants per line) were taken from 5- to 6-week-old  
738 plants grown on soil and incubated in stomatal opening buffer (10 mM MES-KOH, pH 6.15;  
739 50 mM KCl; 10  $\mu\text{M}$  CaCl<sub>2</sub>; 0.01 % Tween-20) for 2 h in a plant growth cabinet in the light.  
740 Subsequently, flg22, AtPep1, ABA or mock were added from stock solutions to the indicated  
741 concentrations and samples incubated under the same conditions for another 2-3 h.  
742 Photographs of the abaxial leaf surface were taken using a Leica DM5500 microscope  
743 equipped with a Leica DFC450 camera. Width and length of the stomatal openings were  
744 determined using the Leica LAS AF software and aperture given as ratio of width divided by  
745 length.

746 Number of stomata counted and underlying statistical analysis in Figure 4 are:

747 Fig. 4b: Col-0 mock: n=346, Col-0 flg22: n=381, *osca1.3* mock: n=382, *osca1.3* flg22: n=435,  
748 *osca1.7* mock: n=435, *osca1.7* flg22: n=448, *osca1.3/1.7* mock: n=460, *osca1.3/1.7* flg22:  
749 n=497. Fig. 4c: Col-0 mock: n=410, Col-0 AtPep1: n=546, Col-0 ABA: n=484, *osca1.3/1.7*  
750 mock: n=477, *osca1.3/1.7* AtPep1: n=520, *osca1.3/1.7* ABA: n=467. Fig. 4f: Col-0 mock:  
751 n=154, Col-0 flg22: n=159, *osca1.3/1.7* mock: n=159, *osca1.3/1.7* flg22: n=181,  
752 *osca1.3/1.7/pOSCA1.3:OSCA1.3(S54A)* mock: n=170,  
753 *osca1.3/1.7/pOSCA1.3:OSCA1.3(S54A)* flg22: n=197, *osca1.3/1.7/pOSCA1.3:OSCA1.3(WT)*  
754 mock: n=108, *osca1.3/1.7/pOSCA1.3:OSCA1.3(WT)* flg22: n=155.

755

### 756 **Gas exchange measurements**

757 Seeds of Col-0 and *osca1.3/1.7* were sown on sterilized soil, and plants were grown in a  
758 climate cabinet with the following conditions: day/night cycle of 12/12 h, temperatures of 21/18  
759 °C, photon flux density of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and relative humidity of 60%. After 12-14 days, the  
760 seedlings were carefully transferred to new pots and grown for another 2-3 weeks, at the same  
761 conditions.

762 Leaf transpiration was recorded with intact leaves, of which the petioles were excised from the  
763 rosette and immediately transferred to distilled water. The petioles were recut twice under  
764 water with a razor blade to avoid embolism, and were quickly transferred into small tubes with  
765 distilled water and wrapped with parafilm. Leaves were placed inside the cuvettes of a custom-  
766 made gas exchange recording system<sup>61</sup>, equipped with two Infra-Red-Gas-Analyzers (IRGA)  
767 (LI 7000; Li-Cor, Lincoln). The air stream through the cuvettes was set to 0.96 l/min and had  
768 a relative humidity of 68% and a CO<sub>2</sub> concentration of 400 ppm. The leaves were illuminated  
769 with LEDs (Cree Xlamp CXA2520 LED) at a photon flux density of 80 μmol m<sup>-2</sup> s<sup>-1</sup>. During the  
770 measurements, stimuli were added to the solution at the petioles to concentrations of 10 μM  
771 flg22, 3 μM AtPep1, 10 μM ABA or 0.01% ethanol (as a control).

772

### 773 **Bacterial spray infection**

774 *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 *COR* strain was grown in overnight culture  
775 in King's B medium supplemented with 50 μg/mL rifampicin, 50 μg/mL kanamycin and 100  
776 μg/mL spectinomycin and incubated at 28 °C. Cells were harvested by centrifugation and  
777 pellets re-suspended in 10 mM MgCl<sub>2</sub> to an OD<sub>600</sub> of 0.2, corresponding to 1x10<sup>8</sup> cfu/mL.  
778 Silwet L77 (Sigma Aldrich) was added to a final concentration of 0.04 %. Four-to-five-week-  
779 old plants (7 to 8 plants per genotype) were sprayed with the suspension and covered with a  
780 lid for three days. Three leaf discs were taken from three leaves per plant and ground in 200  
781 μL water using a 2010 Geno/Grinder (SPEX®SamplePrep LLC, Metuchen, NJ, USA). Serial  
782 dilutions of the extracts were plated on L agar medium containing antibiotics and 25 μg/mL  
783 nystatin. Colonies were counted after incubation at 28 °C for 1.5 to 2 d.

784

### 785 **RNA isolation, cDNA, qRT-PCR**

786 For gene expression analysis, seeds were sown on ½ Murashige and Skoog (MS) medium  
787 (2.2 g/L; including vitamins) supplemented with 1% sucrose and 0.8% agar. Seeds were  
788 stratified for 2 days at 4 °C and incubated for 5 d at 21 °C under a 16-h photoperiod. Seedlings  
789 were then transferred to liquid ½ MS medium with 1% sucrose and grown for another 8 d.  
790 Total RNA was extracted from two seedlings using TRI reagent (Ambion) according to the  
791 manufacturer's instructions. RNA samples were treated with Turbo DNA-free DNase (Ambion)  
792 according to the manufacturer's instructions. RNA was quantified with a Nanodrop  
793 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from RNA using  
794 RevertAid Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's  
795 instructions. cDNA was amplified by quantitative PCR using PowerUp SYBR Green Master  
796 mix (Thermo Fisher Scientific) and an Applied Biosystems 7500 Fast Real-Time PCR System  
797 (Thermo Fisher Scientific). Relative expression values were determined using *U-box*

798 (At5g15400) as a reference and the comparative Ct method ( $2^{-\Delta\Delta C_t}$ ). Primers used are listed  
799 in Supplementary Table 1.

800

### 801 **Statistical analysis**

802 Statistical analysis was performed in GraphPad Prism 7.0. (GraphPad  
803 Software, <http://www.graphpad.com>) unless stated otherwise. Dot plots were used to show  
804 individual data points wherever possible. P values 0.05 were considered non-significant.  
805 Sample sizes, statistical tests used and P values are stated in the figure legends.

806

### 807 **Reporting summary**

808 Further information on research design is available in the Nature Research Reporting  
809 Summary linked to this paper.

810

### 811 **Data availability**

812 For blot source images, see Supplementary Figure 1. Raw data for all graphs are available as  
813 Source Data. All other data or materials can be obtained from the corresponding author upon  
814 request.

815

### 816 **Code availability**

817 All codes used for the wavelet analysis are available at  
818 [https://github.com/TeamMacLean/peak\\_analysis](https://github.com/TeamMacLean/peak_analysis).

819

820

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905

906

907 **Author contributions**

908 C.Z. designed and supervised the project, and obtained funding. K.T. and S.J. conceived,  
909 designed and performed the majority of the plant and biochemical experiments. E.M. and J.F.  
910 provided the patch-clamp data in COS-7 cells; J.G. performed some of the genetic and  
911 phenotypic characterization of the *osca1.3/1.7* mutant. P.D. and F.L.M. performed the SRM  
912 assays. N.L., M.C. and G.E.D.O. provided the yeast complementation assays. K. H. and M.W.  
913 provided the HEK cell data. T.A.D. and J.D. performed aequorin and YC3.6 measurements in  
914 leaf discs. P.K. and J.G. generated expression constructs for OSCA1.7. L.S. assisted with the  
915 genetic characterization of the mutants. Y.K. provided initial data on BIK1-OSCA1.3  
916 interaction. C.A.B. provided OSCA1.3 localization data. S.S., S.H., M.R.G.R. and R.H.  
917 assisted with initial electrophysiological characterization, conducted ion flux measurements  
918 and carried out gas-exchange recordings. D.M. analyzed the guard-cell Ca<sup>2+</sup> measurements.  
919 K.T. and C.Z. wrote the manuscript. All authors commented and agreed on the manuscript  
920 before submission.

921

922

923 **Competing interests**

924 Authors declare no competing interests.

925

926

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930

931

932

933 **Extended Data Figure legends**

934

935 **Extended Data Figure 1 | Predicted topology of OSCA1.3 with possible BIK1**  
936 **phosphorylation sites and multiple alignment of loop 1 from Clade 1 OSCA proteins.**

937 **a** Topology was visualized using Protter ([www.wlab.ethz.ch/protter](http://www.wlab.ethz.ch/protter)) version 1.0 based on  
938 information from Jojoa-Cruz et al. (2018). Blue numbers indicate transmembrane regions.  
939 Possible BIK1 phosphorylation sites are highlighted in red.

940 **b** Protein sequence alignment of OSCA1.1 to OSCA1.8 showing amino acids 30 to 95. Clustal  
941 Omega alignments were visualized with Jalview 2.10.5. Possible BIK1 phosphorylation motifs  
942 (SxxL/I) are highlighted in red. Blue color denotes % identity.

943 **c** Structural model for OSCA1.3. Arrows indicate the position of S54 located in the cytosolic  
944 loop.

945

946 **Extended Data Figure 2 | OSCA1.3 localizes to the plasma membrane.**

947 Confocal microscopy of *osca1.3* cotyledons expressing OSCA1.3-GFP under the control of  
948 the *OSCA1.3* promoter. Right Panel: Plasmolysis with 2 M NaCl underlines plasma membrane  
949 localization. Green: GFP; magenta: chlorophyll autofluorescence.

950

951 **Extended Data Figure 3 | PBL1 also phosphorylates OSCA1.3.**

952 Differences in PBL1-mediated incorporation of radioactive phosphate in OSCA1.3 and its  
953 mutation variants. *In vitro* kinase assay performed with the corresponding recombinant  
954 proteins. For blot source data, see Supplementary Fig.1. The experiment was performed twice  
955 with similar results.

956

957 **Extended Data Figure 4 | OSCA1.3 promotes calcium influx in HEK cells.**

958 HEK293T cells loaded with the calcium indicator Fura-2 and transfected with OSCA1.3-myc  
959 show an increase in fluorescence intensity ratio at 340/380 nm excitation compared to non-  
960 transfected cells after addition of sorbitol and calcium to the culture medium, indicating an  
961 increase in calcium influx. Data show mean  $\pm$  SD (n=4).

962

963 **Extended Data Figure 5 | OSCA1.3 and OSCA1.7 are BIK1-activated calcium-permeable  
964 channels.**

965 **a** Typical currents (left panel) and corresponding I/V curves (right panel) recorded in OSCA1.3  
966 plus BIK1 expressing COS-7 cells increase with increasing calcium concentrations as  
967 indicated on the figure legend (n=3,  $\pm$ SE). Currents were normalized with current intensities  
968 recorded at -100 mV in the standard bath solution (5 mM calcium), and consequently  
969 expressed in normalized arbitrary units for easier comparison of reverse potential changes.  
970 Note the inward currents increase and the reverse potentials shift to positive values when  
971 extracellular calcium concentration increases, indicating a calcium permeation of the channel.  
972 See methods for solutions composition.

973 **b** Typical traces (left panel) and corresponding statistical analysis (right panel) of currents  
974 recorded in whole-cell configuration in COS-7 cells co-transfected with pCI-OSCA1.7 plus pCI-  
975 BIK1 or plus pCI-BIK1-KD as indicated on the figure legend. OSCA1.7 is a BIK1-activated  
976 channel. //V curves recorded on cells.

977 **c** BIK1 kinase activity activates currents in cells expressing both OSCA1.3 and OSCA1.7.  
978 Typical currents (left panel) and corresponding //V curves (right panel) recorded in cells co-

979 transfected with both pCI-OSCA1.3 and pCI-OSCA1.7 plus pCI-BIK1 or plus pCI-BIK1-KD as  
980 indicated on the figure legend. Note that current intensities are not higher than current  
981 intensities recorded in cells expressing either OSCA1.3+BIK1 (Fig. 3b,c) or OSCA1.7+BIK1  
982 (a), giving no indication on functional heteromerization of OSCA1.3 and OSCA1.7. Whole-cell  
983 patch clamp protocols used in b and c were identical to the one used in Fig. 3b,c.

984

985 **Extended Data Figure 6 | T-DNA insertion lines used in this study and transcript levels.**

986 **a** Gene structure of *OSCA1.3* and *OSCA1.7* showing exons (black boxes) and introns (lines)  
987 as well as location of T-DNA insertions. Line *osca1.3/1.7* was obtained by crossing *osca1.3*  
988 and *osca1.7*. Arrows denote location of primers used for genotyping.

989 **b** Transcript levels of *OSCA1.3* and *OSCA1.7* in Col-0, *osca1.3*, *osca1.7* and *osca1.3/1.7* as  
990 determined by quantitative real-time RT-PCR. Values are mean +/- SD (n=6).

991 **c** Transcript levels of *OSCA1.3* in Col-0, *osca1.3/1.7* and *osca1.3/1.7* complemented with  
992 *OSCA1.3(WT)* or *OSCA1.3(S54A)*, respectively. Values are from three independent T1 plants,  
993 n=2 per plant. Shown are quantitative real-time RT-PCR data relative to *U-box* (At5g15400).  
994 Primers used in *b* and *c* are listed in Supplementary Table 1.

995

996 **Extended Data Figure 7 | Expression pattern of OSCA genes from Clade 1.**

997 Tissue-specific expression patterns were obtained from Genevestigator  
998 ([www.genevestigator.com](http://www.genevestigator.com)). *OSCA1.3* shows high expression levels in guard cells and guard  
999 cell protoplasts.

1000

1001 **Extended Data Figure 8 | Flg22-induced calcium influx measured in leaf discs is**  
1002 **comparable between wild-type and *osca1.3/1.7* plants.**

1003 **a** Calcium influx in leaf discs taken of Col-0 and *osca1.3/1.7* plants expressing the calcium  
1004 reporter aequorin. flg22 was added at time point 10 min. Error bars represent mean ± SD  
1005 (n=12). The experiment was performed twice with similar results.

1006 **b** Average values of FRET ratio changes in leaf discs of Col-0 and *osca1.3/1.7* expressing the  
1007 ratiometric calcium reporter YC3.6 obtained in plate reader-based assays. Error bars show  
1008 SE, n = 90 (Col-0) and 47 (*osca1.3/1.7*). The experiment was performed twice with similar  
1009 results.

1010

1011 **Extended Data Figure 9 | Flg22-induced calcium fluxes in *osca1.3/1.7* guard cells are**  
1012 **reduced compared to wild-type guard cells.**

1013 **a** Typical flg22-induced spiking patterns and their distribution in Col-0 and *osca1.3/1.7* guard  
1014 cells. Legends show ratio changes of the Yellow Cameleon 3.6 calcium reporter observed

1015 over time (flg22 added at time point 10 min, indicated by an arrow). The pattern of every cell  
1016 (n=64 for wild-type and n=61 for *osca1.3/1.7*) was assigned to one of the categories based on  
1017 visual assessment.

1018 **b** Left panel, net calcium fluxes of a representative Col-0 and *osca1.3/1.7* guard cell,  
1019 respectively, measured using Scanning Ion Selective Electrodes (SISE). Right panel,  
1020 integrated calcium fluxes over 7 min after addition of flg22 are reduced in *osca1.3/1.7*  
1021 compared to Col-0 (n=29 cells for Col-0, n=23 cells for *osca1.3/1.7*; error bars represent mean  
1022  $\pm$  SEM; bootstrapped Welch two sample t-test, P=0.0464.)

1023 **c** Left panel, flg22-induced calcium fluxes are blocked by lanthanum. Representative calcium  
1024 fluxes measured using Scanning Ion Selective Electrodes (SISE) of Col-0 guard cells with or  
1025 without lanthanum pre-treatment (1 mM lanthanum applied 10 min before flg22 treatment).  
1026 One micromolar flg22 was added at timepoint 0 to epidermal strips. Right panel, integrated  
1027 calcium fluxes over 8 min after addition of flg22 are significantly blocked by lanthanum in Col-  
1028 0 (n=8 without lanthanum and n=5 with lanthanum; error bars represent mean  $\pm$  SEM;  
1029 bootstrapped Welch two sample t-test, P=0.0026).

1030

1031 **Extended Data Figure 10 | AtPep1-induced decrease in stomatal conductance is**  
1032 **impaired in *osca1.3/1.7*.**

1033 Leaf transpiration was recorded in excised intact leaves. AtPep1 was added to the solution at  
1034 the petioles to a concentration of 3  $\mu$ M, water was used as control. Data show mean  $\pm$  SEM  
1035 for n=8-11.

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1038 **Extended Data Table legends**

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1040 **Extended Data Table 1 | Specific transitions used for selected reaction monitoring**  
1041 **(SRM) with OSCA1.3 and control peptide.**

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1044 **Supplementary Information**

1045 This file contains Supplementary Figure 1: Source data for images for gels and blots. Original  
1046 source images for all data obtained by SDS-PAGE, western blots, autoradiography scans and  
1047 Coomassie Blue stained blots and gels; Supplementary Table 1: Primers used in this study.

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