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Bowden, Brislinger-Engelhardt, Hansen et al. – Foxi1 in mucociliary development
 Foxi1 regulates multiple steps of mucociliary development

and ionocyte specification through transcriptional and

epigenetic mechanisms 2
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6 **Foxi1 regulates multiple steps of mucociliary development
and ionocyte specification through transcriptional and
epigenetic mechanisms**
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and ionocyte specification through transcriptional and epigenetic mechanisms

S Sarah Bowden^{1,2,3,8}, Magdalena Maria Brislinger-Engelhardt^{1,2,4,8}, Mona Hansen^{1,4,8},

Africa Temporal-Plo^{1,2,3}, Damian Weber^{1,2}, S **epigenetic mechanisms**
5 Sarah Bowden^{1,2,3,8}, Magdalena
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7 Litwin^{2,5}, Clemens Kreutz^{2,5}, Pete
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Bowden, Britain et al. – Foxin in much states in mu
Bowden et al. – Foxin in much states in much state

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38
39 37 **Abstract**
38 Foxi1 is a
39 subtypes
40 epithelia. 38 Foxi1 is a master regulator of ionocytes (ISCs / INCs) across species and organs. Two
39 subtypes of ISCs exist, and both α - and β-ISCs regulate pH- and ion-homeostasis in
40 epithelia. Gain and loss of FOXI1 funct 39 subtypes of ISCs exist, and both α - and β-ISCs regulate pH- and ion-homeostasis in
40 epithelia. Gain and loss of FOXI1 function are associated with human diseases,
41 including Pendred syndrome, male infertility, epithelia. Gain and loss of FOXI1 function are associated with human diseases,
including Pendred syndrome, male infertility, renal acidosis and cancers. Foxi1
functions were predominantly studied in the context of ISC spec including Pendred syndrome, male infertility, renal acidosis and cancers. Foxi1

functions were predominantly studied in the context of ISC specification, however,

reports indicate additional functions in early and ectode functions were predominantly studied in the context of ISC specification, however,
reports indicate additional functions in early and ectodermal development. Here, we re-
investigated the functions of Foxi1 in *Xenopus lae* reports indicate additional functions in early and ectodermal development. Here, we re-
investigated the functions of Foxi1 in *Xenopus laevis* embryonic mucociliary epidermis
development and found a novel function for Fox investigated the functions of Foxi1 in *Xenopus laevis* embryonic mucociliary epidermis
development and found a novel function for Foxi1 in the generation of Notch-ligand
expressing mucociliary multipotent progenitors (MPP development and found a novel function for Foxi1 in the generation of Notch-ligand
expressing mucociliary multipotent progenitors (MPPs). We demonstrate that Foxi1 has
multiple concentration-dependent functions: At low lev expressing mucociliary multipotent progenitors (MPPs). We demonstrate that Foxi1 has
multiple concentration-dependent functions: At low levels, Foxi1 confers ectodermal
competence through transcriptional and epigenetic mec multiple concentration-dependent functions: At low levels, Foxi1 confers ectodermal
competence through transcriptional and epigenetic mechanisms, while at high levels,
Foxi1 induces a multi-step process of ISC specificatio competence through transcriptional and epigenetic mechanisms, while at high levels,

Foxi1 induces a multi-step process of ISC specification and differentiation. We further

describe how *foxi1* expression is affected thro Foxi1 induces a multi-step process of ISC specification and differentiation. We further
describe how *foxi1* expression is affected through auto- and Notch-regulation, how
Ubp1 and Dmrt2 regulate ISC subtype differentiatio describe how *foxi1* expression is affected through auto- and Notch-regulation, how

51 Ubp1 and Dmrt2 regulate ISC subtype differentiation, and how this developmental

52 program affects Notch signaling as well as mucocil Ubp1 and Dmrt2 regulate ISC subtype differentiation, and how this developmental
program affects Notch signaling as well as mucociliary patterning. Together, we reveal
novel functions for Foxi1 in *Xenopus* mucociliary epid 52 program affects Notch signaling as well as mucociliary patterning. Together, we reveal
53 novel functions for Foxi1 in *Xenopus* mucociliary epidermis formation, relevant to our
54 understanding of vertebrate developmen 53 novel functions for Foxi1 in *Xenopus* mucociliary epidermis formation, relevant to our
154 understanding of vertebrate development and human disease.
155 54 understanding of vertebrate development and human disease.
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Bowden, Britain et al. – Foxin in mucociniary development of al. – Foxin in mucociniary developm

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61 59 **Introduction**
60 The Forkhead
61 the lung, kidn
62 Pou Casellas The Forkhead-box transcription factor Foxi1 is a master regulator of ionocytes (ISCs) in

the lung, kidney, inner ear and epididymis across vertebrates (Hulander et al., 2003;

Pou Casellas et al., 2023). ISCs regulate ion the lung, kidney, inner ear and epididymis across vertebrates (Hulander et al., 2003;
Fou Casellas et al., 2023). ISCs regulate ion homeostasis through the expression of an
Fou Casellas et al., 2023). ISCs regulate ion hom 62 Pou Casellas et al., 2023). ISCs regulate ion homeostasis through the expression of an
63 array of transmembrane solute carriers (e.g. Pendrin encoded by *slc26a4* and Anion
64 exchanger 1 encoded by *slc4a1*) and pH-r array of transmembrane solute carriers (e.g. Pendrin encoded by *slc26a4* and Anion
exchanger 1 encoded by *slc4a1*) and pH-regulators (e.g. vacuolar (v)H⁺ATPase
encoded by *atp6* subunit genes and Carbonic anhydrase e exchanger 1 encoded by *slc4a1*) and pH-regulators (e.g. vacuolar (v)H+ exchanger 1 encoded by *slc4a1*) and pH-regulators (e.g. vacuolar (v)H⁺ATPase
encoded by *atp6* subunit genes and Carbonic anhydrase encoded by *ca* genes). α-ISCs
and β-ISCs are subtypes with apical vs. basolateral vH encoded by *atp6* subunit genes and Carbonic anhydrase encoded by *ca* genes). α -ISCs
and β -ISCs are subtypes with apical vs. basolateral vH⁺ATPase localization and
differential transporter expression of *slc26a4* and β-ISCs are subtypes with apical vs. basolateral vH⁺ATPase localization and and β -ISCs are subtypes with apical vs. basolateral vH⁺ATPase localization and
differential transporter expression of slc26a4 and slc4a1 (Quigley et al., 2011). In ISCs
of the mammalian airway mucociliary epithelium, 68 of the mammalian airway mucociliary epithelium, Foxi1 also regulates the expression of
cystic fibrosis transmembrane conductance regulator (*CFTR*), thereby controlling
chloride secretion and mucus properties (Montoro 69 cystic fibrosis transmembrane conductance regulator (CFTR), thereby controlling
70 chloride secretion and mucus properties (Montoro et al., 2018; Plasschaert et al., 2018;
71 Scudieri et al., 2020). Mutations in *FOXI1* 69 chloride secretion and mucus properties (Montoro et al., 2018; Plasschaert et al., 2018;
69 Scudieri et al., 2020). Mutations in *FOXI1* and its transcriptional target solute carriers
672 cause Pendred svndrome and hear chloride secretion and mucus properties (Montoro et al., 2018; Plasschaert et al., 2018;

Scudieri et al., 2020). Mutations in *FOXI1* and its transcriptional target solute carriers

cause Pendred syndrome and hearing loss 71 Scudieri et al., 2020). Mutations in *FOXI1* and its transcriptional target solute carriers
72 cause Pendred syndrome and hearing loss, male infertility, and distal renal tubular
73 acidosis (Blomqvist et al., 2004; Blo 73 acidosis (Blomqvist et al., 2004; Blomqvist et al., 2006; Hulander et al., 2003; Yang et
174 al., 2007). In contrast, Foxi1 overexpression is found in cancer subtypes, e.g. in
175 chromophore renal cell carcinoma (chRCC 174 al., 2007). In contrast, Foxi1 overexpression is found in cancer subtypes, e.g. in

175 chromophore renal cell carcinoma (chRCC) and in pulmonary large cell carcinoma

176 (LCC) (Lindgren et al., 2017; Simbolo et al., al., 2007). In contrast, Foxi1 overexpression is found in cancer subtypes, e.g. in
chromophore renal cell carcinoma (chRCC) and in pulmonary large cell carcinoma
(LCC) (Lindgren et al., 2017; Simbolo et al., 2024; Skala et chromophore renal cell carcinoma (chRCC) and in pulmonary large cell carcinoma
76 (LCC) (Lindgren et al., 2017; Simbolo et al., 2024; Skala et al., 2020; Yamada et al.,
77 2022).
78 The *Xenopus* embryonic epidermis is a p

(LCC) (Lindgren et al., 2017; Simbolo et al., 2024; Skala et al., 2020; Yamada et al.,
2022).
The *Xenopus* embryonic epidermis is a popular model for vertebrate mucociliary
19 epithelia development and disease studies (Wa 77 2022).
78 The X
79 epitheli The *Xenopus* embryonic epidermis is a popular model for vertebrate mucociliary

epithelia development and disease studies (Walentek and Quigley, 2017). It forms

3 79 epithelia development and disease studies (Walentek and Quigley, 2017). It forms

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Foxi1-dependent α -ISCs and β -ISCs as well as multiciliated cells (MCCs), secretory

cells (small secretory cells (SSCs) and goblet cel 80 Foxi1-dependent α-ISCs and β-ISCs as well as multiciliated cells (MCCs), secretory
81 cells (small secretory cells (SSCs) and goblet cells) and basal stem cells, similar to the
82 mammalian airway epithelium (Brooks an cells (small secretory cells (SSCs) and goblet cells) and basal stem cells, similar to the

mammalian airway epithelium (Brooks and Wallingford, 2014; Haas et al., 2019; Hayes

et al., 2007; Quigley et al., 2011; Walentek, 82 mammalian airway epithelium (Brooks and Wallingford, 2014; Haas et al., 2019; Hayes
83 et al., 2007; Quigley et al., 2011; Walentek, 2022; Walentek et al., 2014). Knockdown of
84 foxi1 in Xenopus caused a loss of epider 84 *foxi1* in *Xenopus* caused a loss of epidermal ISCs as expected, but also induced
85 defective ciliation in MCCs suggesting potential Foxi1 functions in other cell types
86 (Dubaissi and Papalopulu, 2011; Mir et al., 2 foxi1 in *Xenopus* caused a loss of epidermal ISCs as expected, but also induced
defective ciliation in MCCs suggesting potential Foxi1 functions in other cell types
(Dubaissi and Papalopulu, 2011; Mir et al., 2007). Foxi1 defective ciliation in MCCs suggesting potential Foxi1 functions in other cell types

(Dubaissi and Papalopulu, 2011; Mir et al., 2007). Foxi1 plays additional early roles in
 Xenopus ectoderm development: In the blastul 86 (Dubaissi and Papalopulu, 2011; Mir et al., 2007). Foxi1 plays additional early roles in
87 *Xenopus* ectoderm development: In the blastula and gastrula, *foxi1* is initially activated
88 throughout the entire ectoderm *Xenopus* ectoderm development: In the blastula and gastrula, *foxi1* is initially activated
throughout the entire ectoderm where it is required as a determinant counteracting
vegetal mesendoderm-inducing factors (Mir et a 88 throughout the entire ectoderm where it is required as a determinant counteracting
89 vegetal mesendoderm-inducing factors (Mir et al., 2007; Suri et al., 2005). In neurula
80 stages, Foxi1 is required for placode forma 89 vegetal mesendoderm-inducing factors (Mir et al., 2007; Suri et al., 2005). In neurula
80 stages, Foxi1 is required for placode formation in the neural plate border and for the
81 specification of ISCs in the epidermis stages, Foxi1 is required for placode formation in the neural plate border and for the
91 specification of ISCs in the epidermis (Dubaissi and Papalopulu, 2011; Maharana and
92 Schlosser, 2018; Quigley et al., 2011).
93 Ho

specification of ISCs in the epidermis (Dubaissi and Papalopulu, 2011; Maharana and
92 Schlosser, 2018; Quigley et al., 2011).
93 How Foxi1 regulates different aspects of *Xenopus* mucociliary epidermis development
94 from Schlosser, 2018; Quigley et al., 2011).
93 How Foxi1 regulates different aspects
94 from ectoderm to ISC-subtype specifi
95 additional mucociliary epidermis cell 93 How Foxi1 regulates different aspects of *Xenopus* mucociliary epidermis development
94 from ectoderm to ISC-subtype specification, and how it could affect development of
95 additional mucociliary epidermis cell types r 95 additional mucociliary epidermis cell types remains unresolved. Understanding the
96 range of Foxi1 functions in this important model for mucociliary biology could provide
97 additional information regarding unappreciat additional mucociliary epidermis cell types remains unresolved. Understanding the

196 range of Foxi1 functions in this important model for mucociliary biology could provide

197 additional information regarding unapprecia range of Foxi1 functions in this important model for mucociliary biology could provide
additional information regarding unappreciated Foxi1 functions in development and
human disease.
Here, we re-examined the roles of Foxi

98 human disease.
99 Here, we re-examined the roles of Foxi1 in *Xenopus* mucociliary epidermis
100 development. We found that Foxi1 acts in a concentration-dependent manner: At low 98 human disease.
99 Here, we re-e:
00 development. W
01 levels, Foxi1 m 99 Here, we re-examined the roles of Foxi1 in *Xenopus* mucociliary epidermis
00 development. We found that Foxi1 acts in a concentration-dependent manner: At low
01 levels, Foxi1 marks multipotent mucociliary progenitors development. We found that Foxi1 acts in a concentration-dependent manner: At low
101 levels, Foxi1 marks multipotent mucociliary progenitors (MPPs) and establishes
102 ectodermal identity, at least in part through regulat 101 levels, Foxi1 marks multipotent mucociliary progenitors (MPPs) and establishes
102 ectodermal identity, at least in part through regulation of chromatin accessibility. At high
4 102 ectodermal identity, at least in part through regulation of chromatin accessibility. At high

Bowden, Brislinger-Engelhardt, Hansen et al. – Foxi1 in mucociliary development
levels, Foxi1 induces ISC specification and subtype differentiation through Ubp1 and
Dmrt2. High Foxi1 levels are achieved by positive auto-re 103 levels, Foxi1 induces ISC specification and subtype differentiation through Ubp1 and
104 Dmrt2. High Foxi1 levels are achieved by positive auto-regulation and inhibited by
105 Notch signaling. In this work, we elucidat 104 Dmrt2. High Foxi1 levels are achieved by positive auto-regulation and inhibited by
105 Notch signaling. In this work, we elucidate novel concentration-dependent Foxi1
106 transcriptional and epigenetic functions in muc 105 Notch signaling. In this work, we elucidate novel concentration-dependent Foxi1
106 transcriptional and epigenetic functions in mucociliary development and ISC
107 specification.
108 Results transcriptional and epigenetic functions in mucociliary development and ISC
107 specification.
108 Results
109 Specification.and.differentiation.of.ISCs.is.a.multi-step.process 107 specification.
108 **Results**
109 **Specification**

108 **Results

109 Specific**

110 Previous

111 *(*Quidev **Specification and differentiation of ISCs is a multi-step process**
110 Previous work has defined a core ISC gene set in the *X. laevis* mu
111 (Quigley and Kintner, 2017). We generated epidermal mucocilia
112 animal cap e 110 Previous work has defined a core ISC gene set in the *X. laevis* mucociliary epidermis
111 (Quigley and Kintner, 2017). We generated epidermal mucociliary organoids from
112 animal cap explants and conducted bulk RNA-s 111 (Quigley and Kintner, 2017). We generated epidermal mucociliary organoids from
112 animal cap explants and conducted bulk RNA-sequencing (RNA-seq) analysis of core
113 ISCs gene expression during epidermis development animal cap explants and conducted bulk RNA-sequencing (RNA-seq) analysis of core
113 ISCs gene expression during epidermis development (Brislinger-Engelhardt et al., 2023;
114 Haas et al., 2019; Walentek, 2018). Z-scores o 113 ISCs gene expression during epidermis development (Brislinger-Engelhardt et al., 2023;
114 Haas et al., 2019; Walentek, 2018). Z-scores of normalized counts (TPM) of ISC
115 transcripts were clustered to reveal dynamic 114 Haas et al., 2019; Walentek, 2018). Z-scores of normalized counts (TPM) of ISC
115 transcripts were clustered to reveal dynamic co-expression (**Fig. 1A**). Five clusters
116 clearly separated along developmental time, w transcripts were clustered to reveal dynamic co-expression (Fig. 1A). Five clusters
clearly separated along developmental time, with cluster I being the only set of genes
displaying strong expression during very early and clearly separated along developmental time, with cluster I being the only set of genes
displaying strong expression during very early and late developmental stages, but not
during cell fate specification stages (st. 10-16) displaying strong expression during very early and late developmental stages, but not
118 during cell fate specification stages (st. 10-16). Cluster III contained *foxi1*, the Notch
119 ligand *dll1* and the cell cycle reg during cell fate specification stages (st. 10-16). Cluster II contained *foxi1*, the Notch
119 ligand *dll1* and the cell cycle regulator *gadd45g*. Cluster III contained *ca12* and the
120 transcription factor *ubp1*, whi 119 ligand *dll1* and the cell cycle regulator *gadd45g*. Cluster III contained *ca12* and the
120 transcription factor *ubp1*, which was shown to induce ISC formation upon
121 overexpression in the epidermis (Quigley et a transcription factor *ubp1*, which was shown to induce ISC formation upon
121 overexpression in the epidermis (Quigley et al., 2011). Cluster IV contained multiple
122 transcription factors, including *tfcp2l1*, required f overexpression in the epidermis (Quigley et al., 2011). Cluster IV contained multiple
transcription factors, including *tfcp2l1*, required for ISC formation in mouse kidney
(Werth et al., 2017). Cluster V was dominated by transcription factors, including *tfcp2l1*, required for ISC formation in mouse kidney

(Werth et al., 2017). Cluster V was dominated by *slc26a4* and *atp6*-subunit expression

during later differentiation of ISCs (st. 20 123 (Werth et al., 2017). Cluster V was dominated by *slc26a4* and *atp6*-subunit expression
124 during later differentiation of ISCs (st. 20–32). These data indicated that ISCs develop
125 using a multi-step process. 124 during later differentiation of ISCs (st. 20–32). These data indicated that ISCs develop
125 using a multi-step process.
5 125 using a multi-step process.

125 using a multi-step process.

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To validate the core ISC gene set, we used manipulations to deplete or enrich ISCs in
mucociliary organoids and RNA-seq at the beginning of ce 126 To validate the core ISC gene set, we used manipulations to deplete or enrich ISCs in

127 mucociliary organoids and RNA-seq at the beginning of cell fate specification (st. 10), at

128 the end of cell fate specificat mucociliary organoids and RNA-seq at the beginning of cell fate specification (st. 10), at
the end of cell fate specification (st. 16), during maturation and intercalation of ISCs (st.
25), and in the mature epithelium (st the end of cell fate specification (st. 16), during maturation and intercalation of ISCs (st.

25), and in the mature epithelium (st. 32) (Walentek and Quigley, 2017). As previously

130 described (Quigley et al., 2011), i 25), and in the mature epithelium (st. 32) (Walentek and Quigley, 2017). As previously
described (Quigley et al., 2011), increased Notch signaling (Notch intracellular domain
(*nicd*) mRNA injections) inhibited core ISC ge described (Quigley et al., 2011), increased Notch signaling (Notch intracellular domain

131 (nicd) mRNA injections) inhibited core ISC gene expression, while inhibition of Notch

132 signaling (injection of dominant-negat (*nicd*) mRNA injections) inhibited core ISC gene expression, while inhibition of Notch
signaling (injection of dominant-negative suppressor of hairless/RBPJ (*suh-dbm*)
mRNA) promoted core ISC gene expression (**Fig. S1A,B** signaling (injection of dominant-negative suppressor of hairless/RBPJ (*suh-dbm*)
mRNA) promoted core ISC gene expression (**Fig. S1A,B**). Inhibition of Notch signaling
in combination with MCC inhibition (by co-injection of mRNA) promoted core ISC gene expression (Fig. S1A,B). Inhibition of Notch signaling
134 in combination with MCC inhibition (by co-injection of *dominant-negative mcidas* (*dn-*
135 mcidas) mRNA (Stubbs et al., 2012)) furth in combination with MCC inhibition (by co-injection of *dominant-negative mcidas* (*dn-*
 mcidas) mRNA (Stubbs et al., 2012)) further increased core ISC gene expression,

reflecting stronger overproduction of ISCs. Howev mcidas) mRNA (Stubbs et al., 2012)) further increased core ISC gene expression,
reflecting stronger overproduction of ISCs. However, expression of *tfcp2l1*, atp6v0d1.L
and csta.L were reduced in these conditions suggestin reflecting stronger overproduction of ISCs. However, expression of *tfcp2l1*, *atp6v0d1.L*
and *csta.L* were reduced in these conditions suggesting that they might be expressed
more in MCCs than in ISCs (Fig. S1C). Notch r and *csta.L* were reduced in these conditions suggesting that they might be expressed
138 more in MCCs than in ISCs (**Fig. S1C**). Notch repression of *foxi1* by *nicd* was
139 substantial but not statistically significant more in MCCs than in ISCs (**Fig. S1C**). Notch repression of *foxi1* by *nicd* was
substantial but not statistically significant (**Fig. S1A**), potentially reflecting a Notch-
independent function of Foxi1.
We used single-ce

substantial but not statistically significant (**Fig. S1A**), potentially reflecting a Notch-
independent function of Foxi1.
141 We used single-cell RNA-seq (scRNA-seq) data from at late *Xenopus laevis* epidermis
142 stages independent function of Foxi1.
141 We used single-cell RNA-seq
142 stages containing mature ISCs
143 of *foxi1, ubp1* and *atp6*-subul 141 We used single-cell RNA-seq (scRNA-seq) data from at late *Xenopus laevis* epidermis

142 stages containing mature ISCs (Aztekin et al., 2019). These data confirmed high levels

143 of *foxi1, ubp1* and *atp6*-subuni stages containing mature ISCs (Aztekin et al., 2019). These data confirmed high levels of <i>foxi1</i>, <i>ubp1</i> and <i>atp6</i>-subunit expression in both ISC subtypes, while <i>slc4a1</i> was specifically expressed in α-ISCs and <i>slc26a4</i> in β-ISCs, as previously described (Fig. S1D). Interestingly, the transcription factor <i>dmrt2</i> (cluster IV) was exclusively expressed 143 of *foxi1*, *ubp1* and *atp6*-subunit expression in both ISC subtypes, while *slc4a1* was

144 specifically expressed in α-ISCs and *slc26a4* in β-ISCs, as previously described (**Fig.**

145 **S1D**). Interestingly, the 144 specifically expressed in α-ISCs and *slc26a4* in β-ISCs, as previously described (**Fig.** 31D). Interestingly, the transcription factor *dmrt2* (cluster IV) was exclusively expressed by α-ISCs (**Fig. 31D**), and loss o 145 **S1D**). Interestingly, the transcription factor *dmrt2* (cluster IV) was exclusively expressed
by α-ISCs (**Fig. S1D**), and loss of kidney α-ISCs was recently reported in *Dmrt2*
knockout mice (Wu et al., 2024). 146 by α-ISCs (**Fig. S1D**), and loss of kidney α-ISCs was recently reported in *Dmrt2*
147 knockout mice (Wu et al., 2024).
6 147 knockout mice (Wu et al., 2024).

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To test if *foxi1, ubp1* and *dmrt2* contribute differentially to ISC-subtype formation, we
knocked down each factor using morpholino oligonuc 148 To test if *foxi1*, *ubp1* and *dmrt2* contribute differentially to ISC-subtype formation, we

149 knocked down each factor using morpholino oligonucleotides (MOs) and analyzed ISC

150 marker expression in the mature knocked down each factor using morpholino oligonucleotides (MOs) and analyzed ISC
150 marker expression in the mature epidermis at st. 30-32 by whole-mount *in situ*
151 hybridization (WMISH). Knockdown of *foxi1* led to a marker expression in the mature epidermis at st. 30-32 by whole-mount *in situ*
151 hybridization (WMISH). Knockdown of *foxi1* led to a loss of pan-ISC marker *atp6v1e1*,
152 which could be rescued and expanded by co- and hybridization (WMISH). Knockdown of *foxi1* led to a loss of pan-ISC marker *atp6v1e1*,
which could be rescued and expanded by co- and over-expression of *foxi1* mRNA,
respectively (Fig. 1B,C). *foxi1* knockdown also stron which could be rescued and expanded by co- and over-expression of *foxi1* mRNA,
153 respectively (Fig. 1B,C). *foxi1* knockdown also strongly reduced expression of *ubp1*,
154 *dmrt2*, *slc24a6* and *slc4a1* (Fig. 1C,D), i respectively (**Fig. 1B,C**). *foxi1* knockdown also strongly reduced expression of *ubp1*,
 dmrt2, *slc24a6* and *slc4a1* (**Fig. 1C,D**), indicating a loss of both ISC subtypes in the

mucociliary epidermis. In contrast, dmrt2, *slc24a6* and *slc4a1* (**Fig. 1C,D**), indicating a loss of both ISC subtypes in the
155 mucociliary epidermis. In contrast, knockdown of *ubp1* specifically reduced expression
156 of the β-ISC marker *slc26a4*, whi 155 mucociliary epidermis. In contrast, knockdown of *ubp1* specifically reduced expression
156 of the β-ISC marker *slc26a4*, while *dmrt2* loss lead to inhibition of α-ISC-specific *slc4a1*
157 expression (**Fig. 1E-H**) 156 of the β-ISC marker *slc26a4*, while *dmrt2* loss lead to inhibition of α-ISC-specific *slc4a1*
157 expression (Fig. 1E-H). Importantly, *ubp1* and *dmrt2* MOs did not abolish pan-ISC
158 expressed *foxi1* and *atp6v1* expression (**Fig. 1E-H**). Importantly, *ubp1* and *dmrt2* MOs did not abolish pan-ISC
158 expressed *foxi1* and *atp6v1e1* (**Fig. 1E-H**).
159 Taken together, these data suggest that Foxi1 determines ISC fate commitment, wh

expressed *foxi1* and *atp6v1e1* (Fig. 1E-H).
159 Taken together, these data suggest that F
160 Ubp1 and Dmrt2 regulate ISC-subtype diffe
161 159 Taken together, these data suggest that Foxi1 determines ISC fate commitment, while
160 Ubp1 and Dmrt2 regulate ISC-subtype differentiation in a multi-step process.
161 **Foxi1 has multiple concentration-dependent funct** Ubp1 and Dmrt2 regulate ISC-subtype differentiation in a multi-step process.
161
Foxi1 has multiple concentration-dependent functions

162
163
164 Foxi1 has multiple concentration-dependent functions
163 In addition to ISC-specification, an earlier role for Foxi1
164 during blastula stages in *Xenopus* was described (Suri
165 experiments investigating ISC formation a 163 In addition to ISC-specification, an earlier role for Foxi1 as ectodermal determinant
164 during blastula stages in *Xenopus* was described (Suri et al., 2005). However, our
165 experiments investigating ISC formation during blastula stages in *Xenopus* was described (Suri et al., 2005). However, our
165 experiments investigating ISC formation after *foxi1* MO did not indicate a loss of
166 epidermal identity.
167 To confirm this, we in

experiments investigating ISC formation after *foxi1* MO did not indicate a loss of
166 epidermal identity.
167 To confirm this, we injected *foxi1* MO together with membrane GFP to identify targeted
168 cells, and analyze 166 epidermal identity.
167 To confirm this, we
168 cells, and analyze
169 epidermis at st. 32 167 To confirm this, we injected *foxi1* MO together with membrane GFP to identify targeted
168 cells, and analyzed cell type composition and morphology in the mature mucociliary
169 epidermis at st. 32 by immunofluorescen cells, and analyzed cell type composition and morphology in the mature mucociliary

169 epidermis at st. 32 by immunofluorescence (IF) confocal microscopy (Walentek, 2018).

17 epidermis at st. 32 by immunofluorescence (IF) confocal microscopy (Walentek, 2018).
7
7

Bowden, Brislinger-Engelhardt, Hansen et al. – Foxi1 in mucociliary development
As previously described, we also observed reduced ciliation in MCCs after *foxi1*
knockdown (Dubaissi and Papalopulu, 2011) as well as strongl 170 As previously described, we also observed reduced ciliation in MCCs after *foxi1*
171 knockdown (Dubaissi and Papalopulu, 2011) as well as strongly reduced ISC and
172 increased MCC numbers, with little effect on secre 2011) as well as strongly reduced ISC and
172 increased MCC numbers, with little effect on secretory cell types (SSCs and goblet
2173 cells) or epidermal identity (**Fig. 2A**). This indicated that *foxi1* knockdown reduced increased MCC numbers, with little effect on secretory cell types (SSCs and goblet
cells) or epidermal identity (**Fig. 2A**). This indicated that *foxi1* knockdown reduced Foxi1
levels enough to inhibit ISC specification, b cells) or epidermal identity (**Fig. 2A**). This indicated that *foxi1* knockdown reduced Foxi1
174 levels enough to inhibit ISC specification, but not strong enough to prevent ectoderm
175 specification.
176 Next, we caused 174 levels enough to inhibit ISC specification, but not strong enough to prevent ectoderm
175 specification.
176 Next, we caused stronger depletion of Foxi1 using higher doses of *foxi1* MO. This
177 treatment induced dela

175 specification.
176 Next, we cau
177 treatment ind
178 previously de 176 Next, we caused stronger depletion of Foxi1 using higher doses of *foxi1* MO. This
177 treatment induced delamination of cells in gastrula stage embryos (**Fig. S2A**), as
178 previously described (Mir et al., 2007), and 177 treatment induced delamination of cells in gastrula stage embryos (Fig. S2A), as
178 previously described (Mir et al., 2007), and frequent formation of skin lesions at st. 32
179 that could be rescued by co-injection o

previously described (Mir et al., 2007), and frequent formation of skin lesions at st. 32
that could be rescued by co-injection of *foxi1* mRNA (**Fig. 2B**).
This suggests that low levels of Foxi1 are sufficient for ectoder that could be rescued by co-injection of *foxi1* mRNA (**Fig. 2B**).
180 This suggests that low levels of Foxi1 are sufficient for ecto
181 Foxi1 levels are required for ISC specification.
182 180 This suggests that low levels of Foxi1 are sufficient for ectoderm identity, while high
181 Foxi1 levels are required for ISC specification.
182 Foxi1 induces multipotent mucociliary progenitors 181 Foxi1 levels are required for ISC specification.
182
183 **Foxi1 induces multipotent mucociliary prog**

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184
185 183 **Foxi1 induces multipotent mucociliary progenitors**
184 Next, we analyzed *foxi1* expression by WMISH. In
185 stages *foxi1* is expressed at low levels in patches throu
186 which start to resolve at st. 12 with individ 184 Next, we analyzed *foxi1* expression by WMISH. In early blastula/gastrula (st. 9/10)
185 stages *foxi1* is expressed at low levels in patches throughout the prospective ectoderm,
186 which start to resolve at st. 12 wi 185 stages *foxi1* is expressed at low levels in patches throughout the prospective ectoderm,
186 which start to resolve at st. 12 with individual cells strongly increasing *foxi1* expression
187 by st. 16, resulting in a which start to resolve at st. 12 with individual cells strongly increasing *foxi1* expression
187 by st. 16, resulting in a salt-and-pepper pattern of individual cells by st. 32, representing
188 individual ISCs (**Fig. S2B** by st. 16, resulting in a salt-and-pepper pattern of individual cells by st. 32, representing

individual ISCs (**Fig. S2B**). Hence, *foxi1* seems to be initially expressed in more

epidermal cells than just in developing I individual ISCs (**Fig. S2B**). Hence, *foxi1* seems to be initially expressed in more
epidermal cells than just in developing ISCs. To test if *foxi1* is expressed broadly in
epidermal multipotent mucociliary progenitors (M 189 epidermal cells than just in developing ISCs. To test if *foxi1* is expressed broadly in
190 epidermal multipotent mucociliary progenitors (MPPs), we generated a fluorescent
190 190 epidermal multipotent mucociliary progenitors (MPPs), we generated a fluorescent

Bowden, Brislinger-Engelhardt, Hansen et al. – Foxi1 in mucociliary development
reporter (foxi1::gfp-utrophin) (Fig. S2C,D) using a previously characterized foxi1
promoter fragment (Cha et al., 2012).
First, we injected em

191 reporter (*foxi1::gfp-utrophin*) (**Fig. S2C,D**) using a previously characterized *foxi1*
192 promoter fragment (Cha et al., 2012).
193 First, we injected embryos with *foxi1::gfp-utrophin* and analyzed reporter activit promoter fragment (Cha et al., 2012).
193 First, we injected embryos with *foxi1*.
194 32 by IF. Indeed, GFP signals were de
195 goblet cells expressed GFP at low le 193 First, we injected embryos with *foxi1::gfp-utrophin* and analyzed reporter activity at st.
194 32 by IF. Indeed, GFP signals were detected in ISCs, MCCs and SSCs, and even some
195 goblet cells expressed GFP at low le 194 32 by IF. Indeed, GFP signals were detected in ISCs, MCCs and SSCs, and even some
195 goblet cells expressed GFP at low levels (**Fig. 2C**). In contrast, a well-characterized
196 Mcidas/Foxj1-regulated promoter constru 195 goblet cells expressed GFP at low levels (Fig. 2C). In contrast, a well-characterized
196 Mcidas/Foxj1-regulated promoter construct (Tasca et al., 2021) driving mScarletl
197 fluorescence (α -tub::mscarletl) was exp Mcidas/Foxj1-regulated promoter construct (Tasca et al., 2021) driving mScarletl

197 fluorescence (α -tub::mscarletl) was expressed predominantly in MCCs (**Fig. S3A,B**).

198 Next, we confirmed that reporter expression fluorescence (*α-tub::mscarletl*) was expressed predominantly in MCCs (**Fig. S3A,B**).
198 Next, we confirmed that reporter expression dynamics resemble endogenous *foxi1*
199 expression patterns during epidermis developme 198 Next, we confirmed that reporter expression dynamics resemble endogenous *foxi1*
199 expression patterns during epidermis development using WMISH (**Fig. 2D, S3C**) and
190 GFP expression by IF (**Fig. 2E, S4A**). Reporter expression patterns during epidermis development using WMISH (Fig. 2D, S3C) and
200 GFP expression by IF (Fig. 2E, S4A). Reporter-driven *gfp* transcripts were detected at
201 st. 9 - 32, starting with non-epithelial low-l 200 GFP expression by IF (**Fig. 2E, S4A**). Reporter-driven *gfp* transcripts were detected at
201 st. 9 - 32, starting with non-epithelial low-level expression at st. 9/10, which increased
202 by st. 12/16 in deep and supe 201 st. 9 - 32, starting with non-epithelial low-level expression at st. 9/10, which increased
202 by st. 12/16 in deep and superficial layer cells, and at st. 32 expression was found
203 predominantly in epithelial layer by st. 12/16 in deep and superficial layer cells, and at st. 32 expression was found
predominantly in epithelial layer cells (Fig. 2D, S3C). GFP-fluorescent cells were
detected from st. 10 onwards, predominantly in deep la predominantly in epithelial layer cells (Fig. 2D, S3C). GFP-fluorescent cells were
detected from st. 10 onwards, predominantly in deep layer cells, but also in some cells
of the outer epithelial layer (Fig. 2E, S4A). Durin detected from st. 10 onwards, predominantly in deep layer cells, but also in some cells

205 of the outer epithelial layer (**Fig. 2E, S4A**). During st. 12-16, an increasing number of

206 cells became GFP(+), including int 205 of the outer epithelial layer (**Fig. 2E, S4A**). During st. 12-16, an increasing number of cells became GFP(+), including intercalating differentiating cells (**Fig. 2E, S4A**). During st. 20-32, the number of GFP(+) cell cells became GFP(+), including intercalating differentiating cells (**Fig. 2E, S4A**). During
207 st. 20-32, the number of GFP(+) cells decreased and fluorescent cells were
208 progressively confined to the epithelial outer 207 st. 20-32, the number of GFP(+) cells decreased and fluorescent cells were
208 progressively confined to the epithelial outer cell layer - however, basal positioned
209 GFP(+) cells were detected even at st. 32 (Fig. 2 progressively confined to the epithelial outer cell layer - however, basal positioned
209 GFP(+) cells were detected even at st. 32 (Fig. 2E, S4A).
210 Together these data support the conclusion that *foxi1* is initially e

209 GFP(+) cells were detected even at st. 32 (Fig. 2E, S4A).
210 Together these data support the conclusion that *foxi1* is
211 during mucociliary development. 210 Together these data support the conclusion that *foxi1* is initially expressed in MPPs
211 during mucociliary development.
212 211 during mucociliary development.
212
Capacitation of the control of the
control of the control of the control of the control of the control of t

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Bowden, Brislinger-Engelhardt, Hansen et al. – Foxi1 in mucociliary development
 Foxi1(+) multipotent progenitors express Notch ligands during cell fate

specification

DII1 expression was assigned to ISCs by Quigley and

Foxi1(+) multipotent progenitors express Notch ligands during cell fate
214 **specification**
215 *DII1* expression was assigned to ISCs by Quigley and colleagues, similar to Foxi(+) cells
216 **in the zebrafish skin and ma** 214 **specification**
215 *DII1* expression
216 in the zebrafis
217 2020; Quigley *Dll1* expression was assigned to ISCs by Quigley and colleagues, similar to Foxi(+) cells
in the zebrafish skin and mammalian kidney (Janicke et al., 2007; Mukherjee et al.,
2020; Quigley and Kintner, 2017). This is suppo in the zebrafish skin and mammalian kidney (Janicke et al., 2007; Mukherjee et al.,
217 2020; Quigley and Kintner, 2017). This is supported by scRNA-seq data from *X.*
218 *tropicalis* development (Briggs et al., 2018) sho 2020; Quigley and Kintner, 2017). This is supported by scRNA-seq data from *X.*

218 *tropicalis* development (Briggs et al., 2018) showing that *dll1* is transiently enriched in

219 differentiating ISCs (Fig. S4B). Howev *tropicalis* development (Briggs et al., 2018) showing that *dll1* is transiently enriched in
219 differentiating ISCs (Fig. S4B). However, another study observed *dll1* expression
220 overlapping with different cell marke differentiating ISCs (**Fig. S4B**). However, another study observed *dll1* expression

220 overlapping with different cell markers during patterning stages in the *Xenopus*

221 epidermis (Cibois et al., 2015). Our temporal overlapping with different cell markers during patterning stages in the *Xenopus*
221 epidermis (Cibois et al., 2015). Our temporal expression analysis indicated that Cluster
222 II contained very early ISC genes, includin epidermis (Cibois et al., 2015). Our temporal expression analysis indicated that Cluster
222 II contained very early ISC genes, including *foxi1* and *dll1*, likely representing the MPPs
223 and early ISC differentiation s

222 II contained very early ISC genes, including *foxi1* and *dll1*, likely representing the MPPs
223 and early ISC differentiation stages (**Fig. 1A**), in line with both published observations.
224 To address if *dll1* (an 223 and early ISC differentiation stages (Fig. 1A), in line with both published observations.
224 To address if *dll1* (and *dlc*; Brislinger-Engelhardt et al., 2023) expression is part of th
225 differentiation program ac 224 To address if *dll1* (and *dlc*; Brislinger-Engelhardt et al., 2023) expression is part of the
225 differentiation program across mucociliary cell types or specific to MPPs and early ISCs,
226 we tested whether master 225 differentiation program across mucociliary cell types or specific to MPPs and early ISCs,

226 we tested whether master transcription factors inducing cell types of the mucociliary

227 epidermis were able to induce N we tested whether master transcription factors inducing cell types of the mucociliary

227 epidermis were able to induce Notch ligands prematurely. We overexpressed *foxi1* for

228 MPPs/ISCs, *mcidas* and *foxj1* for MCC epidermis were able to induce Notch ligands prematurely. We overexpressed *foxi1* for
228 MPPs/ISCs, *mcidas* and *foxj1* for MCCs, *foxa1* for SSCs or $\triangle N$ -*tp63* for basal cells.
229 Only *foxi1* robustly induced *dll1* 228 MPPs/ISCs, *mcidas* and *foxj1* for MCCs, *foxa1* for SSCs or Δ*N-tp63* for basal cells.
229 Only *foxi1* robustly induced *dll1* and *dlc* (Fig. 3A, S4C), and conversely, depletion of
230 Foxi1 prevented *dll1* expre 229 Only *foxi1* robustly induced *dll1* and *dlc* (**Fig. 3A, S4C**), and conversely, depletion of
230 Foxi1 prevented *dll1* expression during cell fate specification stages (**Fig. 3B**). These
231 results suggested that *d* 230 Foxi1 prevented *dll1* expression during cell fate specification stages (Fig. 3B). These
231 results suggested that *dll1* is expressed in *foxi1*(+) MPPs and terminated by cell fate
232 induction of MCCs, SSCs and bas results suggested that *dll1* is expressed in *foxi1*(+) MPPs and terminated by cell fate
induction of MCCs, SSCs and basal cells, but not in ISCs, which maintain *foxi1*
expression.
This raised the question how *dll1* exp induction of MCCs, SSCs and basal cells, but not in ISCs, which maintain *foxi1*
233 expression.
234 This raised the question how *dll1* expression is terminated during ISC differentiation.
235 The *X. tropicalis* scRNA-se

233 expression.
234 This raised
235 The *X. trop* 234 This raised the question how *dll1* expression is terminated during ISC differentiation.
235 The *X. tropicalis* scRNA-seq data revealed that *dll1* enrichment to ISCs is lost once
10 235 The *X. tropicalis* scRNA-seq data revealed that *dll1* enrichment to ISCs is lost once

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ubp1 is expressed (Briggs et al., 2018) (Fig. S4B). To test if Ubp1 terminates dll1

expression, we knocked down ubp1 and analyzed embryos at *ubp1* is expressed (Briggs et al., 2018) (**Fig. S4B**). To test if Ubp1 terminates *dll1*
expression, we knocked down *ubp1* and analyzed embryos at the end of cell fate
specification (st. 16) by WMISH. *foxi1* was maintai expression, we knocked down *ubp1* and analyzed embryos at the end of cell fate
238 specification (st. 16) by WMISH. *foxi1* was maintained and *dll1* expression was
239 prolonged after loss of Ubp1 (**Fig. 3C**). Analysis o 238 specification (st. 16) by WMISH. *foxi1* was maintained and *dll1* expression was
239 prolonged after loss of Ubp1 (**Fig. 3C**). Analysis of cell type composition at st. 32 by IF
240 in *ubp1* morphants further revealed prolonged after loss of Ubp1 (**Fig. 3C**). Analysis of cell type composition at st. 32 by IF
in ubp1 morphants further revealed reduced MCC and SSC numbers as well as
appearance of intercalating cells with ambiguous morphol in *ubp1* morphants further revealed reduced MCC and SSC numbers as well as
241 appearance of intercalating cells with ambiguous morphology, likely representing
242 incompletely differentiated ISCs (Fig. 3D). These results 241 appearance of intercalating cells with ambiguous morphology, likely representing
242 incompletely differentiated ISCs (Fig. 3D). These results demonstrated that
243 manipulating *foxi1* and ISC differentiation leads to incompletely differentiated ISCs (**Fig. 3D**). These results demonstrated that
243 manipulating *foxi1* and ISC differentiation leads to dysregulated Notch dynamics during
244 mucociliary development, which can affect MCCs

manipulating *foxi1* and ISC differentiation leads to dysregulated Notch dynamics during
mucociliary development, which can affect MCCs and other epidermal cell types.
Together, these data demonstrate that Notch ligands ar mucociliary development, which can affect MCCs and other epidermal cell types.

245 Together, these data demonstrate that Notch ligands are expressed by *foxi1*(+)

246 during mucociliary patterning, and that Ubp1-dependen 245 Together, these data demonstrate that Notch ligands are expressed by *foxi1*(+) MPPs
246 during mucociliary patterning, and that Ubp1-dependent differentiation of ISCs or
247 induction of MCC, SSC and basal cell fates during mucociliary patterning, and that Ubp1-dependent differentiation of ISCs or

induction of MCC, SSC and basal cell fates terminate Notch ligand expression.

248
 Eedback- and auto-regulation during ISC specification 247 induction of MCC, SSC and basal cell fates terminate Notch ligand expression.
248
Feedback- and auto-regulation during ISC specification

249
250
251 Feedback- and auto-regulation during ISC specification
250 Feedback regulation of *dll1* by Notch signaling was sugge
251 development (Deblandre et al., 1999). RNA-seq analys
252 expression after Notch manipulations confir Example 150 Feedback regulation of *dll1* by Notch signaling was suggested in *Xenopus* epidermis
251 development (Deblandre et al., 1999). RNA-seq analysis of ligand and receptor
252 expression after Notch manipulations c development (Deblandre et al., 1999). RNA-seq analysis of ligand and receptor
252 expression after Notch manipulations confirmed that gain of Notch signaling suppresses
253 *dll1*, while blocking Notch increases and prolon expression after Notch manipulations confirmed that gain of Notch signaling suppresses

253 *dll1*, while blocking Notch increases and prolongs *dll1* expression (Fig. S1A,B).

254 Knockdown of *dll1* or *notch1* and subse dll1, while blocking Notch increases and prolongs *dll1* expression (Fig. S1A,B).
254 Knockdown of *dll1* or *notch1* and subsequent analysis of cell type composition by IF at
255 st. 32 revealed a strong increase in ISCs, Example 254 Knockdown of *dll1* or *notch1* and subsequent analysis of cell type composition by IF at
255 st. 32 revealed a strong increase in ISCs, which was reversed by co-injection of *nicd*
256 (Fig. S5A), confirming t 255 st. 32 revealed a strong increase in ISCs, which was reversed by co-injection of *nicd*
256 (Fig. S5A), confirming that ISC specification is negatively regulated by Notch signaling
257 through DII1 and Notch1. Furtherm 256 (Fig. S5A), confirming that ISC specification is negatively regulated by Notch signaling
257 through DII1 and Notch1. Furthermore, unilateral knockdown of *dII1* by different (low to
257 11 257 through Dll1 and Notch1. Furthermore, unilateral knockdown of *dll1* by different (low to

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high) MO concentrations, and analysis of cell type markers between the targeted and
untargeted control side at st. 16/17 by WMISH confirmed th high) MO concentrations, and analysis of cell type markers between the targeted and

untargeted control side at st. 16/17 by WMISH confirmed that modifying DII1 levels

sufficed to affect all cell types (Fig. S5B). Togethe untargeted control side at st. 16/17 by WMISH confirmed that modifying DII1 levels
260 sufficed to affect all cell types (Fig. S5B). Together, these data indicate that MPPs,
261 ligand production and ISC specification are

sufficed to affect all cell types (**Fig. S5B**). Together, these data indicate that MPPs,
161 ligand production and ISC specification are negatively regulated by Notch feedback.
162 This raised the question how MPPs can ach ligand production and ISC specification are negatively regulated by Notch feedback.
262 This raised the question how MPPs can achieve high *foxi1* expression levels requ
263 for specification of ISC fate and Ubp1-/Dmrt2-de 262 This raised the question how MPPs can achieve high *foxi1* expression levels required
263 for specification of ISC fate and Ubp1-/Dmrt2-dependent differentiation of mature ISC
264 subtypes. One potential mechanism for 263 for specification of ISC fate and Ubp1-/Dmrt2-dependent differentiation of mature ISC
264 subtypes. One potential mechanism for conferring robust cell fate decisions is positive
265 auto-regulation, and Foxi1 could act subtypes. One potential mechanism for conferring robust cell fate decisions is positive

265 auto-regulation, and Foxi1 could activate its own expression using core Foxi motifs

266 previously identified in the *foxi1* pro auto-regulation, and Foxi1 could activate its own expression using core Foxi motifs

previously identified in the *foxi1* promoter (Cha et al., 2012). To test this, we deleted a

Foxi2 binding region (**Fig. S2C,D**) and ana previously identified in the *foxi1* promoter (Cha et al., 2012). To test this, we deleted a
267 Foxi2 binding region (Fig. S2C,D) and analyzed reporter activity at st. 32, i.e. long after
268 *foxi2* expression is termina 267 Foxi2 binding region (Fig. S2C,D) and analyzed reporter activity at st. 32, i.e. long after

268 *foxi*2 expression is terminated. This strongly decreased reporter activity (Fig. 4A),

269 suggesting that core Foxi mot *foxi2* expression is terminated. This strongly decreased reporter activity (Fig. 4A),
269 suggesting that core Foxi motifs are also used by Foxi1 to maintain its expression
270 through auto-regulation.
271 To verify that

suggesting that core Foxi motifs are also used by Foxi1 to maintain its expression
270 through auto-regulation.
271 To verify that Foxi1 can also activate its own promoter without contributions from Foxi2,
272 we injected 270 through auto-regulation.
271 To verify that Foxi1 can
272 we injected *foxi1::gfp-uti*
273 lacks maternally deposit 271 To verify that Foxi1 can also activate its own promoter without contributions from Foxi2,
272 we injected *foxi1::gfp-utrophin* vegetally to target the prospective mesendoderm, which
273 lacks maternally deposited *fox* we injected *foxi1::gfp-utrophin* vegetally to target the prospective mesendoderm, which
lacks maternally deposited *foxi2* (Cha et al., 2012). Analysis of reporter-only injected
cells (marked by membrane RFP) in hemisecte 273 lacks maternally deposited *foxi2* (Cha et al., 2012). Analysis of reporter-only injected

274 cells (marked by membrane RFP) in hemisected embryos at st. 11 showed no reporter

275 activity in endodermal cells, while cells (marked by membrane RFP) in hemisected embryos at st. 11 showed no reporter
activity in endodermal cells, while co-injection of *foxi1* mRNA led to ectopic activation of
the reporter (Fig 4B).
In summary, Foxi1 induc

activity in endodermal cells, while co-injection of *foxi1* mRNA led to ectopic activation of

276 the reporter (**Fig 4B**).

277 In summary, Foxi1 induces first DII1-expressing MPPs, which increase Notch levels

278 during 276 the reporter (**Fig 4B**).
277 In summary, Foxi1 in
278 during cell fate spe
279 experiencing low Note 277 In summary, Foxi1 induces first DII1-expressing MPPs, which increase Notch levels
278 during cell fate specification stages and terminates ISC production. Progenitors
279 experiencing low Notch levels activate high *fo* 278 during cell fate specification stages and terminates ISC production. Progenitors
279 experiencing low Notch levels activate high *foxi1* expression through auto-regulation to
280 induce ISC fate, and *dll1* expression experiencing low Notch levels activate high *foxi1* expression through auto-regulation to

induce ISC fate, and *dll1* expression is terminated in differentiating ISCs by Ubp1.

12 280 induce ISC fate, and *dll1* expression is terminated in differentiating ISCs by Ubp1.

281

Bowden, Britannia
Boxi1 regulates genome accessibility in the epidermis

282
283
284 Foxi1 regulates genome accessibility in the epidermis
283 We wondered how early Foxi1 expression could have
284 ectodermal development and formation of mucociliary MF
285 at low levels. Besides its effects counteracting me 283 We wondered how early Foxi1 expression could have such a profound impact on
284 ectodermal development and formation of mucociliary MPPs given that it is expressed
285 at low levels. Besides its effects counteracting m ectodermal development and formation of mucociliary MPPs given that it is expressed
285 at low levels. Besides its effects counteracting mesendoderm induction through
286 transcriptional activation of ectodermal genes in e 285 at low levels. Besides its effects counteracting mesendoderm induction through
286 transcriptional activation of ectodermal genes in early *Xenopus* embryos (Suri et al.,
287 2005) Foxi1 has been shown to remain bound transcriptional activation of ectodermal genes in early *Xenopus* embryos (Suri et al.,
287 2005) Foxi1 has been shown to remain bound to condensed chromatin during mitosis,
288 to remodel nucleosome structure and to alter 2005) Foxi1 has been shown to remain bound to condensed chromatin during mitosis,
288 to remodel nucleosome structure and to alter the transcriptional ground state of cells in
289 zebrafish embryos (Yan et al., 2006). This

to remodel nucleosome structure and to alter the transcriptional ground state of cells in

289 zebrafish embryos (Yan et al., 2006). This suggested a pioneer-like function for Foxi1.

290 To test if Foxi1 affects chromatin zebrafish embryos (Yan et al., 2006). This suggested a pioneer-like function for Foxi1.
290 To test if Foxi1 affects chromatin state and genomic accessibility in *Xenopus* epiderm
291 development, we performed assays for t 290 To test if Foxi1 affects chromatin state and genomic accessibility in *Xenopus* epidermal
291 development, we performed assays for transposase-accessible chromatin with
292 sequencing (ATAC-seq) on st. 10 control epide development, we performed assays for transposase-accessible chromatin with
292 sequencing (ATAC-seq) on st. 10 control epidermal organoids and organoids after
293 knockdown of *foxi1*. This revealed a dramatic reduction in sequencing (ATAC-seq) on st. 10 control epidermal organoids and organoids after

293 knockdown of *foxi1*. This revealed a dramatic reduction in accessible chromatin regions

294 after loss of Foxi1 (control: 311,328, *fox* knockdown of *foxi1*. This revealed a dramatic reduction in accessible chromatin regions
after loss of Foxi1 (control: 311,328, *foxi1*MO: 146,640) (**Fig.5A,B**). In Foxi1-depleted
organoids, 53.7% of accessible regions (16 294 after loss of Foxi1 (control: 311,328, *foxi1*MO: 146,640) (**Fig.5A,B**). In Foxi1-depleted
295 organoids, 53.7% of accessible regions (169,077 peaks) were lost, 45.2% were
296 maintained (142,251 peaks), and 1.1% were 295 organoids, 53.7% of accessible regions (169,077 peaks) were lost, 45.2% were
296 maintained (142,251 peaks), and 1.1% were gained (4,389 peaks) (**Fig. 5B**). Next, we
297 investigated which transcription factor binding 296 maintained (142,251 peaks), and 1.1% were gained (4,389 peaks) (Fig. 5B). Next, we
297 investigated which transcription factor binding motifs were enriched in regions lost,
298 maintained or gained after *foxi1* MO. We investigated which transcription factor binding motifs were enriched in regions lost,

298 maintained or gained after *foxi1* MO. We found that motifs for factors with known

1299 functions in *Xenopus* ectodermal developm maintained or gained after *foxi1* MO. We found that motifs for factors with known
1299 functions in *Xenopus* ectodermal development were enriched in regions that lost
1300 accessibility after *foxi1* knockdown: e.g. Tfap 299 functions in *Xenopus* ectodermal development were enriched in regions that lost
200 accessibility after *foxi1* knockdown: e.g. Tfap2a and Tfap2c, Hic1, Rbfox2, Zac1 that
201 regulate neural and neural crest formation accessibility after *foxi1* knockdown: e.g. Tfap2a and Tfap2c, Hic1, Rbfox2, Zac1 that

1301 regulate neural and neural crest formation as well as Tp63, which regulates epidermal

1302 basal stem cells, and Pitx1, required 301 regulate neural and neural crest formation as well as Tp63, which regulates epidermal
302 basal stem cells, and Pitx1, required for cement gland formation (Fig. 5C) (Giudetti et
303 al., 2014; Luo et al., 2005; Ma et a 302 basal stem cells, and Pitx1, required for cement gland formation (**Fig. 5C**) (Giudetti et 303 al., 2014; Luo et al., 2005; Ma et al., 2007; Ray and Chang, 2020; Schweickert et al.,

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2001; Zhang et al., 2006). In contrast, regions that remained open were enriched for
mesendodermal transcription factors (e.g. Gata6, Tbxt, My 2001; Zhang et al., 2006). In contrast, regions that remained open were enriched for
305 mesendodermal transcription factors (e.g. Gata6, Tbxt, MyoD), and regions that gained
306 in accessibility were enriched in pluripote mesendodermal transcription factors (e.g. Gata6, Tbxt, MyoD), and regions that gained
in accessibility were enriched in pluripotency factors (e.g. Brn1, Oct4) (**Fig. 5C**) (Afouda
et al., 2005; Hopwood et al., 1989; Jerabek in accessibility were enriched in pluripotency factors (e.g. Brn1, Oct4) (**Fig. 5C**) (Afouda
et al., 2005; Hopwood et al., 1989; Jerabek et al., 2014; Mistri et al., 2015; Smith et al.,
1991). Together, these data support et al., 2005; Hopwood et al., 1989; Jerabek et al., 2014; Mistri et al., 2015; Smith et al.,
1991). Together, these data support a function for Foxi1 in regulating accessible
chromatin state during ectodermal development i

1991). Together, these data support a function for Foxi1 in regulating accessible

chromatin state during ectodermal development in *Xenopus*.

Finally, we wondered how loss of Foxi1 affects chromatin accessibility in regi chromatin state during ectodermal development in *Xenopus*.
310 Finally, we wondered how loss of Foxi1 affects chromat
311 harboring important genes for mucociliary epidermis develop
312 region around the *krt12.4* (epider 310 Finally, we wondered how loss of Foxi1 affects chromatin accessibility in regions
311 harboring important genes for mucociliary epidermis development. First, we inspected a
312 region around the *krt12.4* (epidermal ke 311 harboring important genes for mucociliary epidermis development. First, we inspected a
312 region around the *krt12.4* (epidermal keratin) gene on chromosome 9/10.S, which
313 revealed strongly reduced accessibility an strategion around the *krt12.4* (epidermal keratin) gene on chromosome 9/10.S, which
revealed strongly reduced accessibility and indicated a loss of epidermal competence
(Fig. 5D) (Wills et al., 2010). Next, we inspected g strategy reduced accessibility and indicated a loss of epidermal competence
314 (Fig. 5D) (Wills et al., 2010). Next, we inspected genomic loci containing genes
315 associated with MPPs (*dll1.L*), ISCs (*ubp1.L* and *dmrt* 314 (Fig. 5D) (Wills et al., 2010). Next, we inspected genomic loci containing genes
315 associated with MPPs (*dll1.L*), ISCs (*ubp1.L* and *dmrt2.S*), and mucociliary
316 development (*foxj1.L* and *tp63.L*) (Deblandre e 315 associated with MPPs (*dll1.L*), ISCs (*ubp1.L* and *dmrt2.S*), and mucociliary
316 development (*foxj1.L* and *tp63.L*) (Deblandre et al., 1999; Haas et al., 2019; Quigley
317 and Kintner, 2017; Quigley et al., 2011). 316 development (*foxj1.L* and *tp63.L*) (Deblandre et al., 1999; Haas et al., 2019; Quigley
317 and Kintner, 2017; Quigley et al., 2011). In all cases, we found reduced accessibility
318 (**Fig. 5D, Fig. S6A-D**). Together, and Kintner, 2017; Quigley et al., 2011). In all cases, we found reduced accessibility

(Fig. 5D, Fig. S6A-D). Together, these data support a broad function for Foxi1 in

regulating ectodermal and mucociliary developmental

318 (**Fig. 5D, Fig. S6A-D**). Together, these data support a broad function for Foxi1 in
1319 regulating ectodermal and mucociliary developmental potential.
1320 In conclusion, Foxi1 regulates chromatin accessibility requir regulating ectodermal and mucociliary developmental potential.
320 In conclusion, Foxi1 regulates chromatin accessibility i
321 development and mucociliary epidermis patterning. This
322 explanation as to how Foxi1 acts as 320 In conclusion, Foxi1 regulates chromatin accessibility required for ectoderm
321 development and mucociliary epidermis patterning. This provides an additional
322 explanation as to how Foxi1 acts as an ectodermal deter 321 development and mucociliary epidermis patterning. This provides an additional
322 explanation as to how Foxi1 acts as an ectodermal determinant in early Xenopus
323 development. 322 explanation as to how Foxi1 acts as an ectodermal determinant in early *Xenopus*
323 development.
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325 **Discussion** 323 development.
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325 **Discussion**

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|
| 325 **Discussion**

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This work revealed that Foxi1 regulates multiple crucial steps during *Xenopus*

mucociliary epidermis development through transcriptional an 326 This work revealed that Foxi1 regulates multiple crucial steps during *Xenopus*
327 mucociliary epidermis development through transcriptional and epigenetic mechanisms.
328 Initially, low-level *foxi1* expression is ac mucociliary epidermis development through transcriptional and epigenetic mechanisms.
328 Initially, low-level *foxi1* expression is activated in the prospective ectoderm by
329 maternally deposited *foxi2* (Cha et al., 201 Initially, low-level *foxi1* expression is activated in the prospective ectoderm by
329 maternally deposited *foxi2* (Cha et al., 2012). This low-level expression is required to
330 regulate chromatin accessibility for pro 329 maternally deposited *foxi*2 (Cha et al., 2012). This low-level expression is required to
330 regulate chromatin accessibility for pro-ectodermal transcription factors (e.g. Tfap2a/c),
331 mucociliary regulators (e.g. regulate chromatin accessibility for pro-ectodermal transcription factors (e.g. Tfap2a/c),
331 mucociliary regulators (e.g. Tp63) as well as mediators of thyroid hormone, retinoic acid
332 and TGFβ signaling (e.g. Thrb, R mucociliary regulators (e.g. Tp63) as well as mediators of thyroid hormone, retinoic acid
and TGF β signaling (e.g. Thrb, Rar-a, Smad4) that were described to regulate
ectodermal development (Cibois et al., 2015; Edri e 332 and TGFβ signaling (e.g. Thrb, Rar-a, Smad4) that were described to regulate
333 ectodermal development (Cibois et al., 2015; Edri et al., 2023; Haas et al., 2019;
334 Hoffman et al., 2007; Tasca et al., 2021). Since ectodermal development (Cibois et al., 2015; Edri et al., 2023; Haas et al., 2019;
334 Hoffman et al., 2007; Tasca et al., 2021). Since baseline *foxi1* expression is Foxi2- but
335 not Notch-dependent, Notch over-activati 334 Hoffman et al., 2007; Tasca et al., 2021). Since baseline *foxi1* expression is Foxi2- but
335 not Notch-dependent, Notch over-activation does not alter ectodermal identity in line
336 with previous reports (Cha et al.

not Notch-dependent, Notch over-activation does not alter ectodermal identity in line
with previous reports (Cha et al., 2012; Deblandre et al., 1999).
Furthermore, our data support previous findings that loss of Foxi1 lea with previous reports (Cha et al., 2012; Deblandre et al., 1999).
337 Furthermore, our data support previous findings that loss of Fox
338 mesendodermal fates, as loci enriched for pro-mesendoderr
339 (e.g. Gata6, Tbxt, My 337 Furthermore, our data support previous findings that loss of Foxi1 leads to acquisition of
338 mesendodermal fates, as loci enriched for pro-mesendodermal transcription factors
339 (e.g. Gata6, Tbxt, MyoD) remain acces mesendodermal fates, as loci enriched for pro-mesendodermal transcription factors

(e.g. Gata6, Tbxt, MyoD) remain accessible in the absence of Foxi1 (Afouda et al.,

2005; Hopwood et al., 1989; Smith et al., 1991). It is (e.g. Gata6, Tbxt, MyoD) remain accessible in the absence of Foxi1 (Afouda et al.,
2005; Hopwood et al., 1989; Smith et al., 1991). It is attractive to speculate that this is
341 possible, because multiple transcription fa 2005; Hopwood et al., 1989; Smith et al., 1991). It is attractive to speculate that this is

341 possible, because multiple transcription factors enriched in the maintained fraction of

342 peaks (e.g. Gata and Sox family possible, because multiple transcription factors enriched in the maintained fraction of
peaks (e.g. Gata and Sox family members) are known factors with pioneer activity (Hou
et al., 2017; Tremblay et al., 2018), which coul

9342 peaks (e.g. Gata and Sox family members) are known factors with pioneer activity (Hou
1343 et al., 2017; Tremblay et al., 2018), which could compensate for the loss of Foxi1.
1344 After inducing ectodermal identity at et al., 2017; Tremblay et al., 2018), which could compensate for the loss of Foxi1.
344 After inducing ectodermal identity at low levels, Foxi1 then further increases it
345 expression through auto-regulation in mucociliar 344 After inducing ectodermal identity at low levels, Foxi1 then further increases its own
345 expression through auto-regulation in mucociliary multipotent progenitors (MPPs),
346 inducing Notch ligand expression (*dll1* expression through auto-regulation in mucociliary multipotent progenitors (MPPs),
inducing Notch ligand expression (*dll1* and *dlc*) required for Notch-mediated mucociliary
cell fate decisions into ISCs, MCCs, secretory c inducing Notch ligand expression (*dll1* and *dlc*) required for Notch-mediated mucociliary
cell fate decisions into ISCs, MCCs, secretory cells and basal stem cells (Brislinger-
Engelhardt et al., 2023). MPPs not exposed cell fate decisions into ISCs, MCCs, secretory cells and basal stem cells (Brislinger-
348 Engelhardt et al., 2023). MPPs not exposed to elevated Notch levels further increase
15 348 Engelhardt et al., 2023). MPPs not exposed to elevated Notch levels further increase

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foxi1 expression through auto-regulation, and high levels of Foxi1 induce ISC fate, in

line with the known role of Foxi1 as master transcrip *foxi1* expression through auto-regulation, and high levels of Foxi1 induce ISC fate, in
350 line with the known role of Foxi1 as master transcription factor for ionocytes across
351 vertebrate tissues (Pou Casellas et al.

line with the known role of Foxi1 as master transcription factor for ionocytes across
351 vertebrate tissues (Pou Casellas et al., 2023).
352 ISC subtype selection and differentiation is a multi-step process. In the *Xenop* vertebrate tissues (Pou Casellas et al., 2023).
352 ISC subtype selection and differentiation is
953 epidermis, Ubp1 is expressed first in both ISC
954 expression in differentiating ISCs. Ubp1 driv 352 ISC subtype selection and differentiation is a multi-step process. In the *Xenopus*
353 epidermis, Ubp1 is expressed first in both ISC subtypes, which terminates Notch ligand
354 expression in differentiating ISCs. U 353 epidermis, Ubp1 is expressed first in both ISC subtypes, which terminates Notch ligand
354 expression in differentiating ISCs. Ubp1 drives differentiation of β-ISCs, while Dmrt2
355 drives α-ISC differentiation. Inte 354 expression in differentiating ISCs. Ubp1 drives differentiation of β-ISCs, while Dmrt2
355 drives α-ISC differentiation. Interestingly, Dmrt2 has recently been shown to be required
356 for α-ISCs in the mouse kidney, drives α-ISC differentiation. Interestingly, Dmrt2 has recently been shown to be required
356 for α-ISCs in the mouse kidney, while not Ubp1, but the related grainyhead-like
357 transcription factor Tfcp2l1 is employed in 356 for α-ISCs in the mouse kidney, while not Ubp1, but the related grainyhead-like
357 transcription factor Tfcp2l1 is employed in mammalian kidney β-ISCs (Quigley et al.,
358 2011; Werth et al., 2017; Wu et al., 2024). 357 transcription factor Tfcp2l1 is employed in mammalian kidney β-ISCs (Quigley et al.,
358 2011; Werth et al., 2017; Wu et al., 2024). This could explain why DII1 expression is
359 terminated in *Xenopus* epidermal ISCs 2011; Werth et al., 2017; Wu et al., 2024). This could explain why Dll1 expression is
terminated in *Xenopus* epidermal ISCs, but remains active in mammalian kidney ISCs
(also called INCs) (Mukherjee et al., 2020; Werth et

terminated in *Xenopus* epidermal ISCs, but remains active in mammalian kidney ISCs
(also called INCs) (Mukherjee et al., 2020; Werth et al., 2017).
361 Similarly to Ubp1, our results suggest that master transcription fact (also called INCs) (Mukherjee et al., 2020; Werth et al., 2017).
361 Similarly to Ubp1, our results suggest that master transc
362 mucociliary epidermal cell types also terminate DII1/Dlc liga
363 Together, this system pro Similarly to Ubp1, our results suggest that master transcription factors for other

1662 mucociliary epidermal cell types also terminate DII1/DIc ligand expression in MPPs.

1663 Together, this system provides a robust Not mucociliary epidermal cell types also terminate Dll1/Dlc ligand expression in MPPs.
363 Together, this system provides a robust Notch feedback-regulated developmental
364 program for mucociliary epidermis development, with 363 Together, this system provides a robust Notch feedback-regulated developmental
364 program for mucociliary epidermis development, with Foxi1 as a central player that acts
365 through transcriptional and epigenetic mech

program for mucociliary epidermis development, with Foxi1 as a central player that acts
365 through transcriptional and epigenetic mechanisms.
366 Finally, our finding that Foxi1 drives an MPP state during mucociliary epid through transcriptional and epigenetic mechanisms.
366 Finally, our finding that Foxi1 drives an MPP s
367 development could serve as a starting point to bet
368 certain cancers, e.g. chromophore renal cell carcinol 366 Finally, our finding that Foxi1 drives an MPP state during mucociliary epidermis
367 development could serve as a starting point to better understand the role of Foxi1 in
368 certain cancers, e.g. chromophore renal cel development could serve as a starting point to better understand the role of Foxi1 in
368 certain cancers, e.g. chromophore renal cell carcinoma (chRCC) and in pulmonary large
369 cell carcinoma (LCC), both highly associat certain cancers, e.g. chromophore renal cell carcinoma (chRCC) and in pulmonary large

cell carcinoma (LCC), both highly associated with Foxi1 activity (Lindgren et al., 2017;

Simbolo et al., 2024; Skala et al., 2020; Yam cell carcinoma (LCC), both highly associated with Foxi1 activity (Lindgren et al., 2017;
370 Simbolo et al., 2024; Skala et al., 2020; Yamada et al., 2022).
16 370 Simbolo et al., 2024; Skala et al., 2020; Yamada et al., 2022).

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580 (Project ID 431984000), by DFG/ANR grant WA3365/4-1, and by the NHLBI thro 579 Programmes (grant WA3365/2-1 and WA3365/5-1), by DFG SFB1453 NephGen (Project ID 431984000), by DFG/ANR grant WA3365/4-1, and by the NHLBI through a Pathway to Independence Award (K99HL127275) to PW; and under Germany' 580 (Project ID 431984000), by DFG/ANR grant WA3365/4-1, and by the NHLBI through a
581 Pathway to Independence Award (K99HL127275) to PW; and under Germany's
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582 Excellence Strategy (CIBSS – EXC-2189 – Project ID 390939984) to PW and CK.
583 **Author contribution:**
585 SB: epigenetics; MMBE: cell fates an

582 Excellence Strategy (CIBSS – EXC-2189 – Project ID 390939984) to PW and CK.
583 **Author contribution:**
585 SB: epigenetics; MMBE: cell fates and Notch; MOH: reporter studies; ATP, DW
586 PW: experimental support; SB, M 584
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588 584 **Author contribution:**

585 SB: epigenetics; MMB

586 PW: experimental su_l

587 analysis and interpretants modeling; PW, SB: b 585 SB: epigenetics; MMBE: cell fates and Notch; MOH: reporter studies; ATP, DW, SH, PW: experimental support; SB, MMBE, MOH, PW: experimental design, planning, analysis and interpretation of data; FL, TL, CK: crucial disc 586 PW: experimental support; SB, MMBE, MOH, PW: experimental design, planning,
587 analysis and interpretation of data; FL, TL, CK: crucial discussion and mathematical
588 modeling; PW, SB: bioinformatics. PW: study desig 587 analysis and interpretation of data; FL, TL, CK: crucial discussion and mathematical
588 modeling; PW, SB: bioinformatics. PW: study design and supervision, coordinating
21 588 modeling; PW, SB: bioinformatics. PW: study design and supervision, coordinating

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collaborative work, manuscript preparation with input from all authors. SB, MMBE, MOH
contributed equally and can list themselves as first co-
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603 Material and Mathods:

599 Animal experiments:

600 Wild-type *Xenopus laevi*

601 (EXRC) at University of

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604 recommendations provide 599 Animal experiments:

600 Wild-type *Xenopus I*

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605 NXR (RRID:SCR_01

606 RRID:SCR_003280) Wild-type *Xenopus laevis* were obtained from the European Xenopus Resource Centre

(EXRC) at University of Portsmouth, School of Biological Sciences, UK, or Xenopus 1,

USA. Frog maintenance and care was conducted accordi 601 (EXRC) at University of Portsmouth, School of Biological Sciences, UK, or Xenopus 1,
602 USA. Frog maintenance and care was conducted according to standard procedures in
603 the AquaCore facility, University Freiburg, USA. Frog maintenance and care was conducted according to standard procedures in
603 the AquaCore facility, University Freiburg, Medical Center (RI_00544) and based on
604 recommendations provided by the international Xeno the AquaCore facility, University Freiburg, Medical Center (RI_00544) and based on

recommendations provided by the international Xenopus community resource centers

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605 NXR (RRID:SCR_013731) and EXRC as well as by Xenbase (http://www.xenbase.org/,

606 RRID:SCR_003280)(Fisher et al., 2023). This work was NXR (RRID:SCR_013731) and EXRC as well as by Xenbase (http://www.xenbase.org/,

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608 22/43 by the state of Baden-Württemberg.
610 <u>Data ava</u> 607 German animal protection laws and was approved under Registrier-Nr. G-18/76 and G-
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610 <u>Data availability:</u>
611 NGS datasets are available via NCBI GEO, ATAC-seq datasets (# 22/43 by the state of Baden-Württemberg.
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610 Data availability:
611 NGS datasets are available via NCBI GEO
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615 <u>peter walentek@</u>
616 611 NGS datasets are available via NCBI GEO, ATAC-seq datasets (# pending), mRNA-seq
612 datasets were generated in previous studies (GSE130448, GSE215373, GSE215419,
613 GSE262944)(Brislinger-Engelhardt et al., 2023; Haas datasets were generated in previous studies (GSE130448, GSE215373, GSE215419,
613 GSE262944)(Brislinger-Engelhardt et al., 2023; Haas et al., 2019). Imaging and
614 quantification data are available to the scientific commu 613 GSE262944)(Brislinger-Engelhardt et al., 2023; Haas et al., 2019). Imaging and
614 quantification data are available to the scientific community upon request to
615 <u>peter.walentek@medizin.uni-freiburg.de</u>.
618 <u>Manipu</u> quantification data are available to the scientific community upon request to
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616 Manipulation of *Xenopus* Embryos:
618 *X. laevis* eggs were collected and in vitro-fertilized

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619 standard procedures (Sive et al., 2007b;
620 Morpholino oligonucleotides (MOs, Gene
621 eight---
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621 Manipulation of *Xenopus* Embryos:
618 X. *laevis* eggs were collected and
619 standard procedures (Sive et al., 2
620 Morpholino oligonucleotides (MOs,
621 eight-cell stage using a PicoSpritze

- 618 *X. laevis* eggs were collected and in vitro-fertilized, then cultured and microinjected by
619 standard procedures (Sive et al., 2007b; Sive et al., 2010). Embryos were injected with
620 Morpholino oligonucleotides (M 619 standard procedures (Sive et al., 2007b; Sive et al., 2010). Embryos were injected with
620 Morpholino oligonucleotides (MOs, Gene Tools), mRNAs or plasmid DNA at two-cell to
621 eight-cell stage using a PicoSpritzer s
- 620 Morpholino oligonucleotides (MOs, Gene Tools), mRNAs or plasmid DNA at two-cell to
621 eight-cell stage using a PicoSpritzer setup in 1/3x Modified Frog Ringer's solution (MR)
22 621 eight-cell stage using a PicoSpritzer setup in 1/3x Modified Frog Ringer's solution (MR)

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with 2.5% Ficoll PM 400 (GE Healthcare, #17-0300-50), and were transferred after
injection into 1/3x MR containing Gentamycin. Drop size was c

with 2.5% Ficoll PM 400 (GE Healthcare, #17-0300-50), and were transferred after

injection into 1/3x MR containing Gentamycin. Drop size was calibrated to about 7–8nL

per injection.

Embryos injected with hormone-induci injection into 1/3x MR containing Gentamycin. Drop size was calibrated to about 7–8nL
624 per injection.
625 Embryos injected with hormone-inducible constructs of (GFP- ΔN -tp63-GR and MCI-GR)
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631 Embryos injected with hormone-inducible constructs of (GFP-ΔN-tp63-GR and MCI-GR)
627 (Haas et al., 2019; Stubbs et al., 2012) were treated with 10μM Dexamethasone
628 (Sigma-Aldrich/Merck #D4902) in ethanol from eight-ce 627 (Haas et al., 2019; Stubbs et al., 2012) were treated with 10 μ M Dexamethasone
628 (Sigma-Aldrich/Merck #D4902) in ethanol from eight-cell stage until fixation. Ultrapure
629 Ethanol (NeoFroxx #LC-8657.3) was used a

628 (Sigma-Aldrich/Merck #D4902) in ethanol from eight-cell stage until fixation. Ultrapure
629 Ethanol (NeoFroxx #LC-8657.3) was used as vehicle control.
630 Morpholino oligonucleotides (MOs) were obtained from Gene Tool

Ethanol (NeoFroxx #LC-8657.3) was used as vehicle control.
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631 Morpholino oligonucleotides (MOs) were obtained from G
632 *dmrt2, foxi1, notch1* and *ubp1* and were used at doses as indi
633 mRNAs encoding *nicd* (100 631
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 637 631 Morpholino oligonucleotides (MOs) were obtained from Gene Tools targeting *dll1*,
632 *dmrt2, foxi1, notch1* and *ubp1* and were used at doses as indicated in the list below.
633 mRNAs encoding *nicd* (100 ng/µl) (Deb 632 *dmrt2*, *foxi1*, *notch1* and *ubp1* and were used at doses as indicated in the list below.
633 mRNAs encoding *nicd* (100 ng/µl) (Deblandre et al., 1999), *foxi1* (25-100ng/µl) (
635 study using primers listed below 634
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639 634 mRNAs encoding *nicd (*100 ng/μl) (Deblandre et al., 1999), *foxi1 (25-100*ng/μl) (this study using primers listed below into pCS107), *mcidas* (100ng/μl) (Stubbs et al., 2012), *foxi1* (100ng/μl) (Stubbs et al., 2008 635 study using primers listed below into pCS107), *mcidas* (100ng/µl) (Stubbs et al., 2012), *foxj1* (100ng/µl) (Stubbs et al., 2008), *foxa1* (100ng/µl) (Dubaissi et al., 2014; Walentek et al., 2014), ΔN -*tp63* (100n 636 *foxj1* (100ng/μl) (Stubbs et al., 2008), *foxa1* (100ng/μl) (Dubaissi et al., 2014; Walentek

et al., 2014), ΔN-tp63 (100ng/μl) (Haas et al., 2019) were injected together with
 membrane-gfp or *membrane-rfp* (at 5 et al., 2014), Δ*N-tp63* (100ng/μl) (Haas et al., 2019) were injected together with
638 *membrane-gfp* or *membrane-rfp* (at 50ng/μL) or *h2b-rfp* (at 30ng/μL) as lineage tracers.
639 All mRNAs were prepared using the mMe *membrane-gfp* or *membrane-rfp* (at 50ng/μL) or *h2b-rfp* (at 30ng/μL) as lineage tracers.

All mRNAs were prepared using the mMessage Machine kit using Sp6 (Invitrogen

#AM1340) supplemented with RNAse Inhibitor (Promeg 639 All mRNAs were prepared using the mMessage Machine kit using Sp6 (Invitrogen #AM1340) supplemented with RNAse Inhibitor (Promega #N251B).
641
642 The *foxi1::gfp-utrophin, foxi1∆1::gfp-utrophin* and *a-tub::mscarletl*

440 #AM1340) supplemented with RNAse Inhibitor (Promega #N251B).
641 The *foxi1::gfp-utrophin, foxi1∆1::gfp-utrophin* and *a-tub::mscar.*
643 purified using the Pure Yield midiprep kit (Promega #A2492) and injo
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655 foxit.S reporter construct cloning and experiments:

To generate the *foxit.S::gfp-utrophin* reporter cor

from *X. laevis* using the phenol/chloroform DNA p

and associated protocol). A 2.7 kb fragment (Fig

cloned using 651 To generate the *foxi1.S::gfp-utrophin* reporter construct, genomic DNA was prepared
652 from *X. laevis* using the phenol/chloroform DNA purification (ThermoFisher #15593031
653 and associated protocol). A 2.7 kb frag

from *X. laevis* using the phenol/chloroform DNA purification (ThermoFisher #15593031

and associated protocol). A 2.7 kb fragment (Fig.S2D) of the *foxi1.S* promoter was

cloned using Easy-A Hi-Fi Cloning Enzyme (Agilent 653 and associated protocol). A 2.7 kb fragment (Fig.S2D) of the *foxi1.S* promoter was
654 cloned using Easy-A Hi-Fi Cloning Enzyme (Agilent #600404) and primers listed in the
655 table below. The PCR fragment was ligated 654 cloned using Easy-A Hi-Fi Cloning Enzyme (Agilent #600404) and primers listed in the
655 table below. The PCR fragment was ligated using the pGEM-T Easy Vector System
23 table below. The PCR fragment was ligated using the pGEM-T Easy Vector System

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(Promega #A1360). The *foxi1.S* promoter sequence was subclorutrophin (used in (Tasca et al., 2021)) after removal of the *a-tub* using HiFi DNA Assembly (NEB #E2621S) and Q5 High-Fidelity DN. #M0491S) kits. *foxi141::gfp-*656 (Promega #A1360). The *foxi1.S* promoter sequence was subcloned into *a-tub::gfp-*
657 *utrophin* (used in (Tasca et al., 2021)) after removal of the *a-tub* promoter sequence
658 using HiFi DNA Assembly (NEB #E2621S) *utrophin* (used in (Tasca et al., 2021)) after removal of the *a-tub* promoter sequence

using HiFi DNA Assembly (NEB #E2621S) and Q5 High-Fidelity DNA Polymerase (NEB

#M0491S) kits. *foxi141::gfp-utrophin* reporter vers 658 using HiFi DNA Assembly (NEB #E2621S) and Q5 High-Fidelity DNA Polymerase (NEB #M0491S) kits. $foxi1\Delta1::gfp-utrophin$ reporter version (Fig. S2C,D) was generated using Q5 High-Fidelity DNA Polymerase and primers listed in th #M0491S) kits. *foxi1∆1::gfp-utrophin* reporter version (Fig. S2C,D) was generated
660 using Q5 High-Fidelity DNA Polymerase and primers listed in the table below. The *a-
661 tub::mscarletl* reporter was generated by rep 660 using Q5 High-Fidelity DNA Polymerase and primers listed in the table below. The *a-*

661 *tub::mscarletl* reporter was generated by replacing the *gfp-utrophin* sequence in *a-*

662 *tub::gfp-utrophin* by the *mscar* tub::mscarletl reporter was generated by replacing the *gfp-utrophin* sequence in *a*-

662 *tub::gfp-utrophin* by the *mscarletl* sequence using HiFi DNA Assembly and Q5 High-

663 Fidelity DNA Polymerase and primers list *tub::gfp-utrophin* by the *mscarletI* sequence using HiFi DNA Assembly and Q5 High-

Fidelity DNA Polymerase and primers listed below. Final construct sequences were

analyzed by whole-plasmid nanopore sequencing.

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673 668 Whole mount *in situ* hybridization and sections:
669 For antisense *in situ* hybridization probes, *slc2*
670 were cloned from whole-embryo cDNAs derive
671 primers listed below (ISH-primers). All sequeno
672 In addit 669 For antisense *in situ* hybridization probes*, slc26a4, slc4a1, ubp1* and *dmrt2* fragments
670 were cloned from whole-embryo cDNAs derived from stages between 3 and 30 using
671 primers listed below (ISH-primers). All were cloned from whole-embryo cDNAs derived from stages between 3 and 30 using

figures primers listed below (ISH-primers). All sequences were verified by Sanger sequencing.

In addition, the following, previously publish 671 primers listed below (ISH-primers). All sequences were verified by Sanger sequencing.
672 In addition, the following, previously published probes were used: *foxi1* (Quigley et al.,
673 2011), *foxi1* and *mcidas* (St In addition, the following, previously published probes were used: *foxi1* (Quigley et al., 2011), *foxi1* and *mcidas* (Stubbs et al., 2008; Stubbs et al., 2012), *foxa1* (Walentek et al., 2014), *tp63* (Haas et al., 2019

673 2011), *foxj1* and *mcidas* (Stubbs et al., 2008; Stubbs et al., 2012), *foxa1* (Walentek et al., 2014), *tp63* (Haas et al., 2019), *atp6v1e1* (Walentek et al., 2015) and *dll1* (Tasca et al., 2021).
676 Embryos were al., 2014), *tp63* (Haas et al., 2019), *atp6v1e1* (Walentek et al., 2015) and *dll1* (Tasca et al., 2021).

675 al., 2021).

676 Embryos were fixed in MEMFA (100mM MOPS pH7.4, 2mM EGTA, 1mM MgSO4, 3.7% (v/v) Formaldehyde) 675 al., 2021).
676 Embryos w
678 (v/v) Form
679 DNAs wer
680 linearized
681 polymeras
681 polymeras - - - -
677
678
680
681
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683 677 Embryos were fixed in MEMFA (100mM MOPS pH7.4, 2mM EGTA, 1mM MgSO4, 3.7% (v/v) Formaldehyde) overnight at 4°C and stored in 100% Ethanol at -20°C until used.

DNAs were purified using the PureYield Midiprep kit (Prome 678 (v/v) Formaldehyde) overnight at 4°C and stored in 100% Ethanol at -20°C until used.
679 DNAs were purified using the PureYield Midiprep kit (Promega #A2492) and were
680 linearized before in vitro synthesis of anti-s 679 DNAs were purified using the PureYield Midiprep kit (Promega #A2492) and were
680 linearized before in vitro synthesis of anti-sense RNA probes using T7 or Sp6
681 polymerase (Promega, #P2077 and #P108G), RNAse inhibi 680 linearized before in vitro synthesis of anti-sense RNA probes using T7 or Sp6 polymerase (Promega, #P2077 and #P108G), RNAse inhibitor and dig-labeled rNTPs (Roche, #3359247910 and 11277057001). Embryos were in situ h 681 polymerase (Promega, #P2077 and #P108G), RNAse inhibitor and dig-labeled rNTPs
682 (Roche, #3359247910 and 11277057001). Embryos were in situ hybridized according to
683 (Harland, 1991), bleached after staining with B 682 (Roche, #3359247910 and 11277057001). Embryos were in situ hybridized according to
683 (Harland, 1991), bleached after staining with BM Purple (Roche #11442074001) and
684 imaged. Sections were made after embedding in 683 (Harland, 1991), bleached after staining with BM Purple (Roche #11442074001) and
684 imaged. Sections were made after embedding in gelatin-albumin with Glutaraldehyde at
685 50-70µm as described in (Walentek et al., 2 684 imaged. Sections were made after embedding in gelatin-albumin with Glutaraldehyde at
685 50-70μm as described in (Walentek et al., 2012).
686
Probe cloning primers (5'-3'):
Name Sequence
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 $\begin{array}{c} 689 \ 690 \ 691 \ 693 \ 694 \ 695 \end{array}$

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693
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696 Evaluation of WMISH staining and morphological evaluations:

691 Embryos were staged according to Nieuwkoop and Faber (1994) Normal Table of

692 *Xenopus laevis* (Daudin). Garland Publishing Inc, New York ISBN 0-8153-1896 Embryos were staged according to Nieuwkoop and Faber (1994) Normal Table of
692 *Xenopus laevis* (Daudin). Garland Publishing Inc, New York ISBN 0-8153-1896-0. For
693 the foxi1 expression stage series wt embryos from mult *Xenopus laevis* (Daudin). Garland Publishing Inc, New York ISBN 0-8153-1896-0. For
the foxi1 expression stage series wt embryos from multiple batches were mixed and at
least 5 embryos per stage were assessed.
Images of em the *foxi1* expression stage series wt embryos from multiple batches were mixed and at

least 5 embryos per stage were assessed.

Images of embryos after *in situ* hybridization and corresponding sections were imaged

usin least 5 embryos per stage were assessed.
695 Images of embryos after *in situ* hybridizati
696 using a Zeiss AxioZoom setup, Zeiss
697 Axiocam208-color, and images were adjust
698 using Adobe Photoshop.
699 In Fig.1B,C exp Images of embryos after *in situ* hybridization and corresponding sections were imaged

using a Zeiss AxioZoom setup, Zeiss Axiolmager.Z1 or Zeiss Stemi508 with

Axiocam208-color, and images were adjusted for color balance

using a Zeiss AxioZoom setup, Zeiss Axiolmager.Z1 or Zeiss Stemi508 with
697 Axiocam208-color, and images were adjusted for color balance, brightness and contrast
698 using Adobe Photoshop.
700 In Fig.1B,C expression stren Axiocam208-color, and images were adjusted for color balance, brightness and contrast

698 using Adobe Photoshop.

700 In Fig.1B,C expression strength were categorized in normal, reduced, strongly reduced

701 or increased 698 using Adobe Photoshop.
699 In Fig.1B,C expression st
701 or increased. In Fig. 1D-C
702 uninjected control sides a
703 embryos, while depicted
704 expression was scored. In
705 as normal or less (numbe 700
701
702
703
705
705 In Fig.1B,C expression strength were categorized in normal, reduced, strongly reduced

701 or increased. In Fig. 1D-G, 3C, S5B, expression level differences observed between the

702 uninjected control sides and manipulate or increased. In Fig. 1D-G, 3C, S5B, expression level differences observed between the

1702 uninjected control sides and manipulated sides of embryos were scored in whole mount

1703 embryos, while depicted sections are s uninjected control sides and manipulated sides of embryos were scored in whole mount

703 embryos, while depicted sections are shown for clarity. In Fig. 3A and S4C induction of

704 expression was scored. In Fig. 3B *dll1*

embryos, while depicted sections are shown for clarity. In Fig. 3A and S4C induction of

704 expression was scored. In Fig. 3B *dll1* expression in the ventral epidermis was analyzed

705 as normal or less (number of dots expression was scored. In Fig. 3B *dll1* expression in the ventral epidermis was analyzed

705 as normal or less (number of dots and expression intensity).

706 For analyses in Fig.2A and S2A, embryos injected with high do as normal or less (number of dots and expression intensity).

706

707 For analyses in Fig.2A and S2A, embryos injected with h

708 morphology and cell size were evaluated for Fig. S2A (and or

710 in hemisected embryos) a 707
708
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711
712 For analyses in Fig.2A and S2A, embryos injected with high dose of *foxi1* MO, cell

708 morphology and cell size were evaluated for Fig. S2A (and delamination was confirmed

709 in hemisected embryos) and skin lesions wer

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morphology and cell size were evaluated for Fig. S2A (and delamination was confirmed

709 in hemisected embryos) and skin lesions were evaluated for Fig. 2A.

710 Immunofluorescence staining and sample preparation:

712 Wh in hemisected embryos) and skin lesions were evaluated for Fig. 2A.

710

711 **Immunofluorescence staining and sample preparation:**

712 Whole *Xenopus* embryos, were fixed at indicated stages in 4% pa

713 4°C overnight o - 711
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717 Fiam immunofluorescence staining and sample preparation:

712 Whole *Xenopus* embryos, were fixed at indicated sta

713 4°C overnight or 2h at room temperature, then washed

714 PBST (0.1% Triton X-100 in PBS), and were b Whole *Xenopus* embryos, were fixed at indicated stages in 4% paraformaldehyde at

713 4°C overnight or 2h at room temperature, then washed 3x 15min with PBS, 2x 30min in

714 PBST (0.1% Triton X-100 in PBS), and were blo 2713 4°C overnight or 2h at room temperature, then washed 3x 15min with PBS, 2x 30min in

714 PBST (0.1% Triton X-100 in PBS), and were blocked in PBST-CAS (90% PBS

715 containing 0.1% Triton X-100, 10% CAS Blocking; Ther

PBST (0.1% Triton X-100 in PBS), and were blocked in PBST-CAS (90% PBS

containing 0.1% Triton X-100, 10% CAS Blocking; ThermoFischer #00-8120) for 30min-

1h at RT. A detailed protocol was described in (Walentek, 2018).
 715 containing 0.1% Triton X-100, 10% CAS Blocking; ThermoFischer #00-8120) for 30min-
716 1h at RT. A detailed protocol was described in (Walentek, 2018).
717 Mouse anti-Acetylated-a-tubulin (Sigma/Merck #T6793) primary a 16 1h at RT. A detailed protocol was described in (Walentek, 2018).

717

718 Mouse anti-Acetylated-α-tubulin (Sigma/Merck #T6793) primary

719 used to mark cilia / MCCs, Rabbit Anti-serotonin (Sigma/Merck antibody (1:500 - - -
718
719
721
722
723 718 Mouse anti-Acetylated-α-tubulin (Sigma/Merck #T6793) primary antibody (1:1000) was used to mark cilia / MCCs, Rabbit Anti-serotonin (Sigma/Merck #S5545) primary antibody (1:500) was used to mark SSCs applied at 4°C ov 719 used to mark cilia / MCCs, Rabbit Anti-serotonin (Sigma/Merck #S5545) primary

720 antibody (1:500) was used to mark SSCs applied at 4°C overnight. Secondary

721 antibodies AlexaFlour-405-labeled goat anti-mouse (Invi 720 antibody (1:500) was used to mark SSCs applied at 4°C overnight. Secondary

721 antibodies AlexaFlour-405-labeled goat anti-mouse (Invitrogen # A30104) and

722 AlexaFlour 405-labeled goat anti-rabbit antibody (Invitr 721 antibodies AlexaFlour-405-labeled goat anti-mouse (Invitrogen # A30104) and
722 AlexaFlour 405-labeled goat anti-rabbit antibody (Invitrogen #A31556) were used for 2 h
723 at RT (1:250). Antibodies were applied in 100% 722 AlexaFlour 405-labeled goat anti-rabbit antibody (Invitrogen #A31556) were used for 2 h
723 at RT (1:250). Antibodies were applied in 100% CAS Blocking (ThermoFischer #00-
25 723 at RT (1:250). Antibodies were applied in 100% CAS Blocking (ThermoFischer #00-

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8120). Actin was stained by incubation (30-120 min at room temperature) with
AlexaFluor 405-labeled Phalloidin (1:800 in PBSt; Invitrogen #A30 724 8120). Actin was stained by incubation (30-120 min at room temperature) with
725 AlexaFluor 405-labeled Phalloidin (1:800 in PBSt; Invitrogen #A30104), mucus-like
726 compounds were stained by incubation (overnight at 725 AlexaFluor 405-labeled Phalloidin (1:800 in PBSt; Invitrogen #A30104), mucus-like
726 compounds were stained by incubation (overnight at 4°C) with AlexaFluor-594- or -647-
727 labeled or PNA (1:500-1000 in PBSt; Molecu

compounds were stained by incubation (overnight at 4°C) with AlexaFluor-594- or -647-

727 labeled or PNA (1:500-1000 in PBSt; Molecular Probes #L32459 and #L32460).

738 Eluorescence imaging, image processing and analysis 727 labeled or PNA (1:500-1000 in PBSt; Molecular Probes #L32459 and #L32460).
728
729 Eluorescence imaging, image processing and analysis:
731 Confocal imaging was conducted using either a Zeiss LSM880 or a Zeiss L
732 mi ---
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735 730
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735 Fluorescence imaging, image processing and analysis:

731 Confocal imaging was conducted using either a Zei:

732 microscope and Zeiss Zen software in the LIC and B

733 images were adjusted for channel brightness/cont

73 731 Confocal imaging was conducted using either a Zeiss LSM880 or a Zeiss LSM980 microscope and Zeiss Zen software in the LIC and BiMiC imaging facilities. Confocal images were adjusted for channel brightness/contrast, Z-s microscope and Zeiss Zen software in the LIC and BiMiC imaging facilities. Confocal

733 images were adjusted for channel brightness/contrast, Z-stack projections were

734 generated and cell types were quantified based on 733 images were adjusted for channel brightness/contrast, Z-stack projections were
734 generated and cell types were quantified based on their morphology using ImageJ
735 (Schindelin et al., 2012). For analyses in Fig. 3D 734 generated and cell types were quantified based on their morphology using ImageJ

735 (Schindelin et al., 2012). For analyses in Fig. 3D and S5A a detailed protocol for

736 quantification of *Xenopus* epidermal cell ty

(Schindelin et al., 2012). For analyses in Fig. 3D and S5A a detailed protocol for

quantification of *Xenopus* epidermal cell type composition was published (Walentek,

737 2018).

For analysis and comparison of fluoresce quantification of *Xenopus* epidermal cell type composition was published (Walentek,

737 2018).

738 For analysis and comparison of fluorescent reporter construct activity on confocal

740 micrographs (Fig. 4A) in ImageJ, 737 2018).

738 For an

740 microg

741 function

742 imaged

743 scored

744 gastrul: 739
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745 For analysis and comparison of fluorescent reporter construct activity on confocal

740 micrographs (Fig. 4A) in ImageJ, z-projections were performed using the "sum-slices"

741 function. Induction of reporter expression i micrographs (Fig. 4A) in ImageJ, z-projections were performed using the "sum-slices"

function. Induction of reporter expression in the endoderm (Fig. 4B) embryos were

imaged using a Zeiss Axiolmager.Z1 with Axiocam208-co 741 function. Induction of reporter expression in the endoderm (Fig. 4B) embryos were

742 imaged using a Zeiss Axiolmager. Z1 with Axiocam 208-color camera. Induction was

743 scored as positive when GFP fluorescence was 742 imaged using a Zeiss Axiolmager.Z1 with Axiocam208-color camera. Induction was

743 scored as positive when GFP fluorescence was detected in the vegetal half of the

744 gastrula embryo. In some controls, activity was 743 scored as positive when GFP fluorescence was detected in the vegetal half of the

744 gastrula embryo. In some controls, activity was observed in involuting or animally

745 positioned mesoderm, where maternal *foxi2* 9744 gastrula embryo. In some controls, activity was observed in involuting or animally

745 positioned mesoderm, where maternal *foxi2* deposition occurs. Fluorescent intensities

746 were color-coded using the function "

905 positioned mesoderm, where maternal *foxi2* deposition occurs. Fluorescent intensities

746 were color-coded using the function "lookup tables -> fire" (8 bit) in ImageJ.

747 <u>RNA- and ATAC-sequencing on *Xenopus* muc</u> were color-coded using the function "lookup tables -> fire" (8 bit) in ImageJ.

747

748 RNA- and ATAC-sequencing on *Xenopus* mucociliary organoids and bi

<u>analysis:</u>

Manipulations and bulk mRNA-seq used in this paper w 748
749
751
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754 RNA- and ATAC-sequencing on *Xenopus* mucociliary organoids and bioinformatics

749 analysis:

750 Manipulations and bulk mRNA-seq used in this paper were generated and published

751 here: (Brislinger-Engelhardt et al., 2 749 <u>analysis:</u>
750 Manipula
751 here: (Br
752 published
753 For Fig.
755 L and .S

Manipulations and bulk mRNA-seq used in this paper were generated and published

here: (Brislinger-Engelhardt et al., 2023; Haas et al., 2019). scRNA-seq datasets were

published here: (Aztekin et al., 2019; Briggs et al., 1911 here: (Brislinger-Engelhardt et al., 2023; Haas et al., 2019). scRNA-seq datasets were

19752 published here: (Aztekin et al., 2019; Briggs et al., 2018).

19753 For Fig. 1A, data from (Brislinger-Engelhardt et al., 2 published here: (Aztekin et al., 2019; Briggs et al., 2018).

753

754 For Fig. 1A, data from (Brislinger-Engelhardt et al., 2023

755 L. and .S allo-allels were added, and the resulting matrix

756 per line and galaxy.eu 754
755
756
758
759
760 754 For Fig. 1A, data from (Brislinger-Engelhardt et al., 2023) were used, TPM values from

755 L. and .S allo-allels were added, and the resulting matrix was clustered using Z-values

756 per line and galaxy.eu (ggplot2_h 755 .L and .S allo-allels were added, and the resulting matrix was clustered using Z-values

756 per line and galaxy.eu (ggplot2_heatmap2/3.1.3.1+galaxy). For Fig. S1A-C, log2-fold

757 changes were calculated using galaxy per line and galaxy.eu (ggplot2_heatmap2/3.1.3.1+galaxy). For Fig. S1A-C, log2-fold

changes were calculated using galaxy.eu (DeSeq2/2.1.3+galaxy) and visualized using

(ggplot2_heatmap2/3.1.3.1+galaxy). For Fig. S1 D, the 757 changes were calculated using galaxy.eu (DeSeq2/2.1.3+galaxy) and visualized using (ggplot2_heatmap2/3.1.3.1+galaxy). For Fig. S1 D, the online tool associated with (Aztekin et al., 2019) was used (marionilab.cruk.cam. (ggplot2_heatmap2/3.1.3.1+galaxy). For Fig. S1 D, the online tool associated with

759 (Aztekin et al., 2019) was used (marionilab.cruk.cam.ac.uk/XenopusRegeneration). For

760 Fig. S4B, the online tool associated with (Br (Aztekin et al., 2019) was used (marionilab.cruk.cam.ac.uk/XenopusRegeneration). For

760 Fig. S4B, the online tool associated with (Briggs et al., 2018) was used

761 (kleintools.hms.harvard.edu/tools/currentDatasetsList_ Fig. S4B, the online tool associated with (Briggs et al., 2018) was used

761 (kleintools.hms.harvard.edu/tools/currentDatasetsList_xenopus_v2.html) to extract

762 lineage-enriched transcripts and the heatmap was generate

(kleintools.hms.harvard.edu/tools/currentDatasetsList_xenopus_v2.html) to extract

762 lineage-enriched transcripts and the heatmap was generated using galaxy.eu

763 (ggplot2_heatmap2/3.1.3.1+galaxy).

764 For ATAC-seq sa lineage-enriched transcripts and the heatmap was generated using galaxy.eu

763 (ggplot2_heatmap2/3.1.3.1+galaxy).

764

765 For ATAC-seq sample generation, injected and control embryos were cultured until st.

766 8. Anim (ggplot2_heatmap2/3.1.3.1+galaxy).

764

765 For ATAC-seq sample generation, ir

766 8. Animal caps were dissected in 1x

767 0.5x MBS + Gentamycin (Sive et al

768 were collected in PBS and ATAC-seq

769 2013; Esmaeili et - 765
766
767
768
769 For ATAC-seq sample generation, injected and control embryos were cultured until st.

8. Animal caps were dissected in 1x Modified Barth's solution (MBS) and transferred to

767 0.5x MBS + Gentamycin (Sive et al., 2007a). 766 8. Animal caps were dissected in 1x Modified Barth's solution (MBS) and transferred to

767 0.5x MBS + Gentamycin (Sive et al., 2007a). 2 organoids per condition and replicate

768 were collected in PBS and ATAC-seq wa 0.5x MBS + Gentamycin (Sive et al., 2007a). 2 organoids per condition and replicate

768 were collected in PBS and ATAC-seq was performed as described in (Buenrostro et al.,

769 2013; Esmaeili et al., 2020). In short: Emb 768 were collected in PBS and ATAC-seq was performed as described in (Buenrostro et al., 2020). In short: Embryos were injected bilaterally in the animal 26
2013; Esmaeili et al., 2020). In short: Embryos were injected bil 769 2013; Esmaeili et al., 2020). In short: Embryos were injected bilaterally in the animal

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hemisphere at the two-cell stage with 3pmol *foxi1* MO or remained uninjected, animal

caps were prepared at st. 8, and organoids were colle 770 hemisphere at the two-cell stage with 3pmol *foxi1* MO or remained uninjected, animal

771 caps were prepared at st. 8, and organoids were collected upon the appearance of the

772 dorsal lip in control embryos cultur 771 caps were prepared at st. 8, and organoids were collected upon the appearance of the

772 dorsal lip in control embryos cultured in parallel to the organoids (st. 10). Organoids

773 were transferred from MBS plates i 772 dorsal lip in control embryos cultured in parallel to the organoids (st. 10). Organoids
773 were transferred from MBS plates into a 1.5 mL low-bind microcentrifuge tube
774 (Eppendorf #0030108051) containing 1 mL of i 773 were transferred from MBS plates into a 1.5 mL low-bind microcentrifuge tube (Eppendorf #0030108051) containing 1 mL of ice-cold 1x PBS. Samples were spun at 500 g at 4 °C in the centrifuge for five minutes before rem 774 (Eppendorf #0030108051) containing 1 mL of ice-cold 1x PBS. Samples were spun at 500 g at 4 °C in the centrifuge for five minutes before removing the PBS and repeating the wash step with fresh ice-cold 1x PBS. 50 μ 775 500 g at 4 °C in the centrifuge for five minutes before removing the PBS and repeating

776 the wash step with fresh ice-cold 1x PBS. 50 μ L of ice-cold lysis buffer (10 mM Tris pH

777 7.4, 10 mM NaCl, 3 mM MgCl2, 776 the wash step with fresh ice-cold 1x PBS. 50 μ L of ice-cold lysis buffer (10 mM Tris pH
777 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% (w/v) Igepal CA-630) and pipetted to break up
778 samples. Samples were centrifuged at 5 777 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% (w/v) Igepal CA-630) and pipetted to break up

778 samples. Samples were centrifuged at 500 g for 10 min at 4 °C and pellets were

779 resuspended in 25 µL TD Buffer, 2.5 µL TDE1 Enzy 778 samples. Samples were centrifuged at 500 g for 10 min at 4 $^{\circ}$ C and pellets were

779 resuspended in 25 µL TD Buffer, 2.5 µL TDE1 Enzyme and 22.5 µL Nuclease-Free

780 water (Illumina #20034198). Samples were pelle resuspended in 25 μ L TD Buffer, 2.5 μ L TDE1 Enzyme and 22.5 μ L Nuclease-Free

780 water (Illumina #20034198). Samples were pelleted to mix and incubated on a

781 ThermoMixer at 37 °C, 700 rpm for 30 min. Followi water (Illumina #20034198). Samples were pelleted to mix and incubated on a

781 ThermoMixer at 37 °C, 700 rpm for 30 min. Following incubation, the samples were

782 cleaned with MinElute Reaction Cleanup Kit (Qiagen, #2

ThermoMixer at 37 °C, 700 rpm for 30 min. Following incubation, the samples were

782 cleaned with MinElute Reaction Cleanup Kit (Qiagen, #28204), following manufacturer

783 instructions and eluted into 11 µL Buffer EB.
 cleaned with MinElute Reaction Cleanup Kit (Qiagen, #28204), following manufacturer

783 instructions and eluted into 11 µL Buffer EB.

784 Libraries were prepared in collaboration with the NIG, University Medical Center
 783 instructions and eluted into 11 µL Buffer EB.
784
785 Libraries were prepared in collaboration
786 Göttingen. Quality was assessed with the Ag
787 the ATAC-seq Kit (Active Motif, #53150). Sa
788 Illumina NovaSeq6000 wi 785
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791 Libraries were prepared in collaboration with the NIG, University Medical Center

786 Göttingen. Quality was assessed with the Agilent Fragment Analyzer and prepared with

787 the ATAC-seq Kit (Active Motif, #53150). Sampl

786 Göttingen. Quality was assessed with the Agilent Fragment Analyzer and prepared with

787 the ATAC-seq Kit (Active Motif, #53150). Samples were sequenced in triplicate on an

788 Illumina NovaSeq6000 with 150 nucleotid the ATAC-seq Kit (Active Motif, #53150). Samples were sequenced in triplicate on an

788 Illumina NovaSeq6000 with 150 nucleotide paired-end reads, totaling 50 million reads

789 per sample.

790 Raw sequencing files were Illumina NovaSeq6000 with 150 nucleotide paired-end reads, totaling 50 million reads

per sample.

Raw sequencing files were assessed for quality using FastQC (v0.11.9, Andrews, S.

FastQC A Quality Control tool for High T 789 per sample.

790 Raw seque

791 FastQC A

792 http://www.t

793 were remov

794 were aligne

795 https://arxiv.

795 (v1.21) (Da 790 Raw sequencing files were assessed for quality using FastQC (v0.11.9, Andrews, S.
791 FastQC A Quality Control tool for High Throughput Sequence Data.
792 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and 791 FastQC A Quality Control tool for High Throughput Sequence Data.

792 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapter sequences

793 were removed with TrimGalore (v0.6.7, https://doi.org/10.5281 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapter sequences

793 were removed with TrimGalore (v0.6.7, https://doi.org/10.5281/zenodo.7598955). Data

794 were aligned to the *X. laevis* genome assembl were removed with TrimGalore (v0.6.7, https://doi.org/10.5281/zenodo.7598955). Data

794 were aligned to the *X. laevis* genome assembly v9.2 using BWA-MEM (v0.7.17,

795 https://arxiv.org/abs/1303.3997). Mitochondrial rea were aligned to the *X. laevis* genome assembly v9.2 using BWA-MEM (v0.7.17,
795 https://arxiv.org/abs/1303.3997). Mitochondrial reads were removed using Samtools
796 (v1.21) (Danecek et al., 2021), and peak calling was pe 795 https://arxiv.org/abs/1303.3997). Mitochondrial reads were removed using Samtools (v1.21) (Danecek et al., 2021), and peak calling was performed with the callpeak function of MACS2 (v2.2.7.1) (Feng et al., 2012). Diffe (v1.21) (Danecek et al., 2021), and peak calling was performed with the callpeak
function of MACS2 (v2.2.7.1) (Feng et al., 2012). Differential analysis was performed
with the bdgdiff function of MACS2 and Venn diagrams w function of MACS2 (v2.2.7.1) (Feng et al., 2012). Differential analysis was performed

798 with the bdgdiff function of MACS2 and Venn diagrams were generated with

799 VennDiagram v1.7.3 in R v4.4.1. Heatmaps showing the with the bdgdiff function of MACS2 and Venn diagrams were generated with

799 VennDiagram v1.7.3 in R v4.4.1. Heatmaps showing the average ATAC-seq signal

800 were generated using deepTools (v3.5.4) (Ramírez et al., 2016 VennDiagram v1.7.3 in R v4.4.1. Heatmaps showing the average ATAC-seq signal

were generated using deepTools (v3.5.4) (Ramírez et al., 2016). Peaks were annotated

for the nearest X. laevis gene and transcription factor bi were generated using deepTools (v3.5.4) (Ramírez et al., 2016). Peaks were annotated
for the nearest X. laevis gene and transcription factor binding motifs with Homer (v4.11)
(Heinz et al., 2010), Plant-specific transcript 801 for the nearest *X. laevis* gene and transcription factor binding motifs with Homer (v4.11)
802 (Heinz et al., 2010), Plant-specific transcription factors were manually excluded from the
803 lists of transcription fact (Heinz et al., 2010), Plant-specific transcription factors were manually excluded from the

803 lists of transcription factors.. Bioinformatic analyses were performed on the Galaxy /

804 Europe platform (usegalaxy.eu) (Co lists of transcription factors.. Bioinformatic analyses were performed on the Galaxy /

804 Europe platform (usegalaxy.eu) (Community, 2024). ATAC-seq data generated for this

805 study was deposited at NCBI GEO under (#pe Europe platform (usegalaxy.eu) (Community, 2024). ATAC-seq data generated for this

study was deposited at NCBI GEO under (#pending).

Stacked bar graphs were generated in Microsoft Excel. Heatmaps and Venn diagrams

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study was deposited at NCBI GEO under (#pending).
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<u>Quantification and statistical evaluation:</u>
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- Stacked bar graphs were generated in Microsoft Excel. Heatmaps and Venn diagrams

were generated using the Galaxy Europe platform (usegalaxy.eu) and R.

Sample sizes for all experiments were chosen based on previous experi were generated using the Galaxy Europe platform (usegalaxy.eu) and R.

810 Sample sizes for all experiments were chosen based on previous experiments

811 embryos derived from at least two different females. No randomizati 810 Sample sizes for all experiments were chosen based on previous experience and used
811 embryos derived from at least two different females. No randomization or blinding was
812 applied.
813 Use of shared controls:
814 811 embryos derived from at least two different females. No randomization or blinding was
812 applied.
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814 <u>Use of shared controls:</u>
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814 <u>Use of sl</u> 814
814 814 Use of shared controls:
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840 836 **Figure legends:**
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**838 Figure 1: Foxi1,
839 (A)** Temporal exp
840 scores of TPMs
841 mucociliary organ 838
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843 **Figure 1: Foxi1, Ubp1 and Dmrt2 differentially regulate ionocyte development**
 (A) Temporal expression analysis of core ISC genes. Heatmap of line-normalized z-

scores of TPMs (transcripts per million reads) derived f 839 **(A)** Temporal expression analysis of core ISC genes. Heatmap of line-normalized z-
840 scores of TPMs (transcripts per million reads) derived from mRNA-seq of *Xenopus*
841 mucociliary organoids over the course of de scores of TPMs (transcripts per million reads) derived from mRNA-seq of *Xenopus*
mucociliary organoids over the course of development (st. 9 - 32). **(B-G)** Knockdown of
ISC transcription factors (*foxi1* MO, *ubp1* MO, 841 mucociliary organoids over the course of development (st. 9 - 32). **(B-G)** Knockdown of
842 ISC transcription factors (*foxi1* MO, *ubp1* MO, *dmrt2* MO) and analysis of effects by
843 WMISH at st. 29 - 32 against *a* 842 ISC transcription factors (*foxi1* MO, *ubp1* MO, *dmrt2* MO) and analysis of effects by
843 WMISH at st. 29 - 32 against *atpv1e1* and *foxi1* (pan-ISC markers), *ubp1* and
844 *slc25a4/pendrin* (β-ISC markets), and 843 WMISH at st. 29 - 32 against *atpv1e1* and *foxi1* (pan-ISC markers), *ubp1* and *slc25a4/pendrin* (β -ISC markets), and *dmrt2* and *slc4a1/ae1* (α -ISC markers). Representative images (**B**,D,E,G) and quantificat 844 *slc25a4/pendrin* (β-ISC markets), and *dmrt2* and *slc4a1/ae1* (α-ISC markers).
845 Representative images (B,D,E,G) and quantification (C, F, H) of results. n = number of
846 embryos analyzed per condition. Rescues (845 Representative images **(B,D,E,G)** and quantification **(C, F, H)** of results. n = number of embryos analyzed per condition. Rescues (co-injection of *foxi1* mRNA) depicted in **(B,C)** were scored as normal (norm.), reduc embryos analyzed per condition. Rescues (co-injection of *foxi1* mRNA) depicted in
847 **(B,C)** were scored as normal (norm.), reduced (red.), strongly reduced (s.red.) and
848 increased (incr.). Color code is shown in **(B) (B,C)** were scored as normal (norm.), reduced (red.), strongly reduced (s.red.) and

848 increased (incr.). Color code is shown in **(B)**. In conditions depicted in **(D, E, G)**,

850 expression levels were scored as less, increased (incr.). Color code is shown in **(B)**. In conditions depicted in **(D, E, G)**,
expression levels were scored as less, more or equal expression as compared to the
uninjected control side. Color code is shown in **(F**

expression levels were scored as less, more or equal expression as compared to the

uninjected control side. Color code is shown in (F).

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 Figure 2: Foxi1 acts in a concentration-dependent manner to specify multi

uninjected control side. Color code is shown in **(F)**.
851
852
853 **Figure 2: Foxi1 acts in a concentration-depend
854 progenitors**
855 **(A)** Immunofluorescence confocal micrographs
856 morphants (*foxi1* MO; low concent 851 853
854
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858 **Figure 2: Foxi1 acts in a concentration-dependent manner to specify multipotent progenitors**
 A) Immunofluorescence confocal micrographs (IF) from control (ctrl.) and *foxi1* morphants (*foxi1* MO; low concentration) a 854 **progenitors**

855 **(A)** Immunoi

856 morphants (*f*

857 α-tub., cilia, g

858 magenta). Ta

859 green). Loca 855 **(A)** Immunofluorescence confocal micrographs (IF) from control (ctrl.) and *foxi1* morphants (*foxi1* MO; low concentration) at st. 32 stained for Acetylated-α-tubulin (Ac.- α -tub., cilia, grey), F-actin (Actin, c 856 morphants (*foxi1* MO; low concentration) at st. 32 stained for Acetylated-α-tubulin (Ac.-

857 α-tub., cilia, grey), F-actin (Actin, cell borders and morphology, grey), and mucus (PNA,

858 magenta). Targeted cells w α -tub., cilia, grey), F-actin (Actin, cell borders and morphology, grey), and mucus (PNA, magenta). Targeted cells were identified by membrane GFP expression (memGFP, green). Location of insets is indicated by dashed 858 magenta). Targeted cells were identified by membrane GFP expression (memGFP,
859 green). Location of insets is indicated by dashed yellow box in upper panels.
28 859 green). Location of insets is indicated by dashed yellow box in upper panels.
28

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Quantification of cell type composition is depicted as pie-charts, goblet cells (blue), ISCs

(yellow), MCCs (green) and SSCs (red). n embryo 860 Quantification of cell type composition is depicted as pie-charts, goblet cells (blue), ISCs (yellow), MCCs (green) and SSCs (red). n embryos (above chart) and n quantified cells (in/left of chart). **(B)** Brightfield 861 (yellow), MCCs (green) and SSCs (red). n embryos (above chart) and n quantified cells
862 (in/left of chart). **(B)** Brightfield images and quantification of st. 30-32 embryos.
863 Uninjected controls (ctrl.), *foxi1* (in/left of chart). **(B)** Brightfield images and quantification of st. 30-32 embryos.

863 Uninjected controls (ctrl.), *foxi1* morphants *(foxi1* MO; high concentration) and

864 morphants co-injected with *foxi1* mRNA (863 Uninjected controls (ctrl.), *foxi1* morphants (*foxi1* MO; high concentration) and morphants co-injected with *foxi1* mRNA (rescue) are depicted. Skin lesions (dashed yellow outline) were quantified. (C,D,E) Analysi 864 morphants co-injected with *foxi1* mRNA (rescue) are depicted. Skin lesions (dashed yellow outline) were quantified. (C,D,E) Analysis of *foxi1::gfp-utrophin* reporter (green) injected embryos by IF (C,E) and WMISH a 9865 yellow outline) were quantified. (C,D,E) Analysis of *foxi1::gfp-utrophin* reporter (green)

866 injected embryos by IF (C,E) and WMISH against *gfp* (D). (C) IF for Acetylated-α-tubulin

867 (Ac.-α-tub., cilia, gre 866 injected embryos by IF (C,E) and WMISH against *gfp* (D). (C) IF for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey), F-actin (Actin, cell borders and morphology, grey), and serotonin (SSCs, grey) at st. 32. Targeted 867 (Ac.-α-tub., cilia, grey), F-actin (Actin, cell borders and morphology, grey), and serotonin (SSCs, grey) at st. 32. Targeted cells were identified by nuclear RFP expression (H2B-
869 RFP, blue). Magnifications of in 868 (SSCs, grey) at st. 32. Targeted cells were identified by nuclear RFP expression (H2B-
869 RFP, blue). Magnifications of intercalating GFP(+) cell types are shown in insets.
870 Location of insets is indicated by dash RFP, blue). Magnifications of intercalating GFP(+) cell types are shown in insets.

870 Location of insets is indicated by dashed yellow boxes in left panels. (D) Sections of

871 epidermal locations from embryos depicted 870 Location of insets is indicated by dashed yellow boxes in left panels. **(D)** Sections of epidermal locations from embryos depicted in Fig. S3C show *gfp* expressing cells in the epidermis at key stages of mucociliary d epidermal locations from embryos depicted in Fig. S3C show *gfp* expressing cells in the

872 epidermis at key stages of mucociliary development (st. 10, 16, 25, 32). **(E)** IF for
 foxi1::gfp-utrophin reporter (green) an epidermis at key stages of mucociliary development (st. 10, 16, 25, 32). **(E)** IF for
 foxi1::gfp-utrophin reporter (green) and F-actin (Actin, cell borders and morphology,

magenta) at st. 10.5 - 32 on hemistected embry foxi1::gfp-utrophin reporter (green) and F-actin (Actin, cell borders and morphology,

1874 magenta) at st. 10.5 - 32 on hemistected embryos. Targeted cells were identified by

1875 membrane RFP expression (mRFP, grey). Ad magenta) at st. 10.5 - 32 on hemistected embryos. Targeted cells were identified by

875 membrane RFP expression (mRFP, grey). Additional stages and full images shown in

876 Fig. S4A.

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878 **Figure 3: Foxi1 induces an**

875 membrane RFP expression (mRFP, grey). Additional stages and full images shown in
876 Fig. S4A.
878 **Figure 3: Foxi1 induces and Ubp1 terminates Notch ligand expression**
880 **(A,B,C)** Manipulation of mucociliary cell fa 876 Fig. S4A.
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878 **Figure 3:**
880 **(A,B,C)** N
881 (ISCs/MP
882 analysis c
883 ligand) ar 878
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884 **Figure 3: Foxi1 induces and Ubp1 terminates Notch ligand expression**
 A,B,C) Manipulation of mucociliary cell fate transcription factors *foxi*

(ISCs/MPPs), *mcidas* and *foxi1* (MCCs), *foxa1* (SSCs) and *ΔN-tp63* (ba **(A,B,C)** Manipulation of mucociliary cell fate transcription factors *foxi1* and *ubp1* (ISCs/MPPs), *mcidas* and *foxi1* (MCCs), *foxa1* (SSCs) and $\triangle N$ -*tp63* (basal cells) and analysis of effects by WMISH at st. 9 **(** 881 (ISCs/MPPs), *mcidas* and *foxj1* (MCCs), *foxa1* (SSCs) and Δ*N-tp63* (basal cells) and analysis of effects by WMISH at st. 9 (A), st. 11 (B) and st. 16 (C) against *dll1* (Notch ligand) and *foxi1* (MPP/ISC marker). analysis of effects by WMISH at st. 9 **(A)**, st. 11 **(B)** and st. 16 **(C)** against *dll1* (Notch ligand) and *foxi1* (MPP/ISC marker). **(A)** Representative images of control (ctrl.) and manipulated embryos (animal views) a ligand) and *foxi1* (MPP/ISC marker). **(A)** Representative images of control (ctrl.) and

884 manipulated embryos (animal views) after mRNA overexpression of transcription factors

885 to test premature induction of *dll1* manipulated embryos (animal views) after mRNA overexpression of transcription factors

885 to test premature induction of *dll1*. Quantification of results and effects on *dlc* are shown

in Fig. S4C. Embryos were scored to test premature induction of *dll1*. Quantification of results and effects on *dlc* are shown
in Fig. S4C. Embryos were scored as induced or non-induced expression. (B)
Representative images of control (ctrl.) and *foxi1* 886 in Fig. S4C. Embryos were scored as induced or non-induced expression. **(B)**
887 Representative images of control (ctrl.) and *foxi1* morphants (*foxi1* MO) (ventral views)
888 to test effects on *dll1* expression. Qua Representative images of control (ctrl.) and *foxi1* morphants (*foxi1* MO) (ventral views)
888 to test effects on *dll1* expression. Quantification of results is shown in lower panel.
889 Locations of insets are indicated to test effects on *dll1* expression. Quantification of results is shown in lower panel.

Locations of insets are indicated by dashed yellow box in upper panels. Embryos were

scored as normal or reduced (less) expressio Locations of insets are indicated by dashed yellow box in upper panels. Embryos were

scored as normal or reduced (less) expression of *dll1*. (C) Representative images of

section embryos after unilateral knockdown of *u* scored as normal or reduced (less) expression of *dll1*. (C) Representative images of section embryos after unilateral knockdown of *ubp1* (*ubp1* MO). Expression of markers was scored as more, less or equal to uninjecte section embryos after unilateral knockdown of *ubp1* (*ubp1* MO). Expression of markers
was scored as more, less or equal to uninjected control (ctrl.) side. Locations of insets
are indicated by dashed yellow box in left was scored as more, less or equal to uninjected control (ctrl.) side. Locations of insets
are indicated by dashed yellow box in left panels. (D) IF of control (ctrl.) and *ubp1*
morphants (*ubp1* MO) at st. 32 stained for are indicated by dashed yellow box in left panels. **(D)** IF of control (ctrl.) and *ubp1* morphants (*ubp1* MO) at st. 32 stained for Acetylated- α -tubulin (Ac.- α -tub., cilia, grey), F-actin (Actin, cell borders and 894 morphants (*ubp1* MO) at st. 32 stained for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey),
895 F-actin (Actin, cell borders and morphology, grey), and mucus (PNA, magenta).
896 Targeted cells were identified by membr 895 F-actin (Actin, cell borders and morphology, grey), and mucus (PNA, magenta).
896 Targeted cells were identified by membrane GFP expression (memGFP, green).
897 Location of insets is indicated by dashed yellow box in u Targeted cells were identified by membrane GFP expression (memGFP, green).

897 Location of insets is indicated by dashed yellow box in upper panels. Quantification of

898 cell type composition is depicted as pie-charts, Location of insets is indicated by dashed yellow box in upper panels. Quantification of

898 cell type composition is depicted as pie-charts, goblet cells (blue), ISCs (yellow), MCCs

899 (green) and SSCs (red). n embryos cell type composition is depicted as pie-charts, goblet cells (blue), ISCs (yellow), MCCs

899 (green) and SSCs (red). n embryos (above chart) and n quantified cells (in/left of chart).

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 Figure 4: Foxi1 regulates it

(green) and SSCs (red). n embryos (above chart) and n quantified cells (in/left of chart).

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 Figure 4: Foxi1 regulates its own expression
 (A) IF of embryos injected with *foxi1::gfp-utrophin* or *foxi1∆1::gfp-utr* ---
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905 Figure 4: Foxi1 regulates its own expression (A) IF of embryos injected with $foxi1::gfp-utro$ (green) at st. 32 stained for Acetylated- α -tubulin cell borders and morphology, grey), and seroton **(A)** IF of embryos injected with *foxi1::gfp-utrophin* or *foxi1∆1::gfp-utrophin* reporters (green) at st. 32 stained for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey), F-actin (Actin, cell borders and morphology, grey) 904 (green) at st. 32 stained for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey), F-actin (Actin,

cell borders and morphology, grey), and serotonin (SSCs, grey) at st. 32. Targeted cells

29 905 cell borders and morphology, grey), and serotonin (SSCs, grey) at st. 32. Targeted cells

were identified by nuclear RFP expression (H2B-RFP, blue). Right
color of GFP fluorescence intensity. (B) Brightfield and epifluore
hemisected st. 11 gastrula embryos injected vegetally with *foxi1::g*
membrane RFP (memRFP 906 were identified by nuclear RFP expression (H2B-RFP, blue). Right panels show false-

907 color of GFP fluorescence intensity. (B) Brightfield and epifluorescence images of

908 hemisected st. 11 gastrula embryos injec 907 color of GFP fluorescence intensity. **(B)** Brightfield and epifluorescence images of hemisected st. 11 gastrula embryos injected vegetally with *foxi1::gfp-utrophin* (green), membrane RFP (memRFP; magenta) as control 908 hemisected st. 11 gastrula embryos injected vegetally with *foxi1::gfp-utrophin* (green),
909 membrane RFP (memRFP; magenta) as control (*memRFP*) or with additional co-
910 injection of *foxi1* mRNA (*foxi1* + *memRFP* 909 membrane RFP (memRFP; magenta) as control (*memRFP*) or with additional co-
910 injection of *foxi1* mRNA (*foxi1* + *memRFP*). Right panels show false-color of GFP
911 fluorescence intensity. Induction was scored as p 910 injection of *foxi1* mRNA (*foxi1* + *memRFP*). Right panels show false-color of GFP
911 fluorescence intensity. Induction was scored as positive when GFP was detected in
912 areas below the equator (mesendoderm). Ctrl 911 fluorescence intensity. Induction was scored as positive when GFP was detected in

912 areas below the equator (mesendoderm). Ctrl. n = 7 induced, 26 non-induced; *foxi1*

913 mRNA = 26 induced, 11 non-induced.

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912 areas below the equator (mesendoderm). Ctrl. n = 7 induced, 26 non-induced; *foxi1*
913 mRNA = 26 induced, 11 non-induced.
915
915 **Figure 5: Foxi1 regulates mucociliary epidermal competence through epigenetic
917 me**

913 mRNA = 26 induced, 11 non-induced.
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915 **Figure 5: Foxi1 regulates mucocili
917 means
918 (A)** Profiles of ATAC-Seq normalized
919 (ctrl.) and foxi1 morphant (*foxi1*MO) ---
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921 **Figure 5: Foxi1 regulates mucociliary epidermal competence through epigenetic means**
 (A) Profiles of ATAC-Seq normalized accessibility around peak center \pm 1 Lkb in controls (ctrl.) and foxi1 morphant (foxi1MO) organ 917 **means**

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919 (ctrl.) are

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923 epiderm **(A)** Profiles of ATAC-Seq normalized accessibility around peak center ± 1 Lkb in controls (ctrl.) and foxi1 morphant (foxi1MO) organoids. (B) Venn diagram of peaks present in uninjected organoids (grey) and foxi1 MO-i 919 (ctrl.) and foxi1 morphant (*foxi1NI*O) organoids. (**B)** Venn diagram of peaks present in uninjected organoids (grey) and *foxi1* MO-injected organoids (purple). (**C)** Top 15 transcription factor binding motifs predict 920 uninjected organoids (grey) and *foxi1* MO-injected organoids (purple). (**C)** Top 15 transcription factor binding motifs predicted in sets of peaks with lost, maintained or gained accessibility after *foxi1* MO. (D) D 921 transcription factor binding motifs predicted in sets of peaks with lost, maintained or
922 gained accessibility after *foxi1* MO. (D) Distribution of accessible regions around
923 epidermal genes $krt12.4$ and $dl1$. L 922 gained accessibility after *foxi1* MO. **(D)** Distribution of accessible regions around
923 epidermal genes $krt12.4$ and $dl11$. Lost, maintained and gained tracks as generated by
924 MACS2 bdgdiff analysis and visualiz epidermal genes *krt12.4* and *dll1*. Lost, maintained and gained tracks as generated by

924 MACS2 bdgdiff analysis and visualized in IGV. Turquoise track = control (ctrl.) and

925 purple track = morphant (*foxi1* MO). n 924 MACS2 bdgdiff analysis and visualized in IGV. Turquoise track = control (ctrl.) and
925 purple track = morphant (*foxi1* MO). n = 2 organoids per condition and replicate. 3
927
928 **Figure S1: Notch regulation of ISC**

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purple track = morphant (*foxi1* MO). n = 2 organoids per condition and replicate. 3
926 replicates.
928 **Figure S1: Notch regulation of ISC genes and ISC-subtype markers**
930 **(A,B,C)** Effects of Notch gain (*nicd*; **A**), 926 replicates.

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928 **Figure S1:**

930 **(A,B,C)** Ef

931 loss (suh-c

932 stages (st.

933 DEseq2. A 928
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935 **Figure S1: Notch regulation of ISC genes and ISC-subtype markers**

930 **(A,B,C)** Effects of Notch gain (*nicd*; **A**), Notch loss (*suh-dbm*; **B**) and N

931 loss (*suh-dbm* + *dn-mcidas*; **C**) on core ISC gene expression 930 **(A,B,C)** Effects of Notch gain (*nicd*; **A**), Notch loss (*suh-dbm*; **B**) and Notch and MCC
931 loss (*suh-dbm* + *dn-mcidas*; **C**) on core ISC gene expression in key developmental
932 stages (st. 10, 16, 25, 32). Hea 931 loss (*suh-dbm* + *dn-mcidas*; **C**) on core ISC gene expression in key developmental
932 stages (st. 10, 16, 25, 32). Heatmaps depict log2-fold change values derived from
933 DEseq2. Asterisks indicate statistical sign 932 stages (st. 10, 16, 25, 32). Heatmaps depict log2-fold change values derived from

933 DEseq2. Asterisks indicate statistical significant (adj-p value < 0.05) changes. (D)

934 Boxplots of ISC gene expression from scRN DEseq2. Asterisks indicate statistical significant (adj-p value < 0.05) changes. **(D)**

934 Boxplots of ISC gene expression from scRNA-seq data published in Aztekin et al.,

935 2019. Visualization was generated using the Boxplots of ISC gene expression from scRNA-seq data published in Aztekin et al.,

935 2019. Visualization was generated using the published online tool:

936 marionilab.cruk.cam.ac.uk/XenopusRegeneration.

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938 **Figure**

2019. Visualization was generated using the published online tool:

marionilab.cruk.cam.ac.uk/XenopusRegeneration.

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 Figure S2: Early Foxi1 functions, expression and reporter construct
 (A) Representative brightfie marionilab.cruk.cam.ac.uk/XenopusRegeneration.

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 **Figure S2: Early Foxi1 functions, expression an

(A)** Representative brightfield images of controls (c

941 foxi1 MO (foxi1MO; high concentration) injection a

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945 **Figure S2: Early Foxi1 functions, expression and reporter construct**
 (A) Representative brightfield images of controls (ctrl.) and embryos (anin

foxi1 MO (foxi1MO; high concentration) injection at st. 8. Morphants sh 940 **(A)** Representative brightfield images of controls (ctrl.) and embryos (animal views) after foxi 1 MO (foxi 1 MO; high concentration) injection at st. 8. Morphants showed enlarged cells and delamination of animal cel 941 *foxi1* MO (*foxi1*MO; high concentration) injection at st. 8. Morphants showed enlarged
942 cells and delamination of animal cells into the blastocoel. Quantification of results
943 shown in the right graph. Delamina cells and delamination of animal cells into the blastocoel. Quantification of results

943 shown in the right graph. Delamination events were scored based on morphological

944 analysis. n = number of embryos. (B) WMISH e 943 shown in the right graph. Delamination events were scored based on morphological
944 analysis. n = number of embryos. (B) WMISH expression analysis of *foxi1* across
945 mucociliary epidermis development stages (st. 9 analysis. n = number of embryos. **(B)** WMISH expression analysis of *foxi1* across

mucociliary epidermis development stages (st. 9 - 32). St. 9, 10 = animal views; st. 12,

946 16 = ventral views; st. 25, 32 = lateral vi 945 mucociliary epidermis development stages (st. 9 - 32). St. 9, 10 = animal views; st. 12, 16 = ventral views; st. 25, 32 = lateral views. Bottom row panels = magnified views of epidermal areas. (C,D) Generation and pro 946 16 = ventral views; st. 25, 32 = lateral views. Bottom row panels = magnified views of
947 epidermal areas. (C,D) Generation and promoter sequences of *foxi1::gfp-utrophin* or
948 *foxi141::gfp-utrophin* reporters. (C epidermal areas. **(C,D)** Generation and promoter sequences of *foxi1::gfp-utrophin* or

948 *foxi141::gfp-utrophin* reporters. **(C)** Schematic representation of cloned genomic *foxi1.S*

949 promoter locus (grey box) and p *foxi1∆1::gfp-utrophin* reporters. (C) Schematic representation of cloned genomic *foxi1.S*
promoter locus (grey box) and position of Foxi2 binding region determined in Cha et al.,
2012 (black outlined box). (D) Promoter 949 promoter locus (grey box) and position of Foxi2 binding region determined in Cha et al.,

950 2012 (black outlined box). (D) Promoter sequence with indicated predicted core Foxi

951 binding-motifs (yellow) and Foxi2 b 950 2012 (black outlined box). **(D)** Promoter sequence with indicated predicted core Foxi
951 binding-motifs (yellow) and Foxi2 binding region (bold, underscored).
³⁰ 951 binding-motifs (yellow) and Foxi2 binding region (bold, underscored).

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 Figure S3: Characterization of the foxi1 reporter
 (A,B) IF of embryos injected with *foxi1::gfp-utrophin* (green) and α -tub.::mscarl 953
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959 **Figure S3: Characterization of the foxi1 reporter**

955 **(A,B)** IF of embryos injected with *foxi1::gfp-utrc*

(magenta) reporters at st. 32 stained for Acetylated

957 actin (Actin, cell borders and morphology, grey), a 955 **(A,B)** IF of embryos injected with *foxi1::gfp-utrophin* (green) and α-tub.::mscarletl
956 (magenta) reporters at st. 32 stained for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey), F-
2957 actin (Actin, cell borders 956 (magenta) reporters at st. 32 stained for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey), F-

actin (Actin, cell borders and morphology, grey), and serotonin (SSCs, grey) in (A); or

for Acetylated-α-tubulin (Ac.-α-t actin (Actin, cell borders and morphology, grey), and serotonin (SSCs, grey) in (A); or

for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey) and F-actin (Actin, cell borders and

morphology, grey), in (B). In (B) targeted 958 for Acetylated-α-tubulin (Ac.-α-tub., cilia, grey) and F-actin (Actin, cell borders and morphology, grey), in (B). In (B) targeted cells were identified by nuclear RFP expression (H2B-RFP, magenta). (C) WMISH express 959 morphology, grey), in **(B)**. In **(B)** targeted cells were identified by nuclear RFP expression (H2B-RFP, magenta). **(C)** WMISH expression analysis of *foxi1::gfp-utrophin* (stained for *gfp* transcripts) across mucoci expression (H2B-RFP, magenta). **(C)** WMISH expression analysis of *foxi1::gfp-utrophin* (stained for *gfp* transcripts) across mucociliary epidermis development stages (st. 9 - 32). St. 9, 10 = animal views; st. 12, 16 = v 961 (stained for *gfp* transcripts) across mucociliary epidermis development stages (st. 9 -
962 32). St. 9, 10 = animal views; st. 12, 16 = ventral views; st. 25, 32 = lateral views.
963 Bottom row panels = magnified view 962 32). St. 9, 10 = animal views; st. 12, 16 = ventral views; st. 25, 32 = lateral views.
963 Bottom row panels = magnified views of epidermal areas. Related to sections shown in
964 Fig. 2D.
965 **Figure S4: Foxi1 express**

Bottom row panels = magnified views of epidermal areas. Related to sections shown in

964 Fig. 2D.

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968 **Figure S4: Foxi1 expression in mucociliary development and Notch ligand

968 (A)** IF for *foxi1::gfp-utrop*

964 Fig. 2D.
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966 **Figure**
968 **inductio
969 (A)** IF f
970 morpholo 966
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973 **Figure S4: Foxi1 expression in mucociliary development and Notch ligand

969 (A)** IF for *foxi1::gfp-utrophin* reporter (green) and F-actin (Actin, cell borders and

970 morphology, magenta) at st. 10.5 - 32 on hemistecte 968 **induction**
969 **(A)** IF for
970 morpholog
971 identified t
972 Fig. 2E. **(E**
973 from scRN
974 published
975 kleintools. 969 **(A)** IF for *foxi1::gfp-utrophin* reporter (green) and F-actin (Actin, cell borders and morphology, magenta) at st. 10.5 - 32 on hemistected embryos. Targeted cells were identified by membrane RFP expression (mRFP, g 970 morphology, magenta) at st. 10.5 - 32 on hemistected embryos. Targeted cells were

971 identified by membrane RFP expression (mRFP, grey). Related to sections shown in

972 Fig. 2E. (B) Heatmap of mucociliary marker ge 971 identified by membrane RFP expression (mRFP, grey). Related to sections shown in

972 Fig. 2E. (B) Heatmap of mucociliary marker gene enrichment differentiation inlneages

973 from scRNA-seq data published in Briggs e Fig. 2E. **(B)** Heatmap of mucociliary marker gene enrichment differentiation inlneages
from scRNA-seq data published in Briggs et al., 2018. Values were derived using the
published online tool:
kleintools.hms.harvard.edu/t 973 from scRNA-seq data published in Briggs et al., 2018. Values were derived using the

974 published conline

975 kleintools.hms.harvard.edu/tools/currentDatasetsList_xenopus_v2.html. NNE = non-

976 neural ectodermal p published online tool:

Relations.harvard.edu/tools/currentDatasetsList_xenopus_v2.html. NNE = non-

975 heural ectodermal precursors; BC – basal cells; ISC = ionocytes; MCC = multiciliated

977 cells; SSC = small secretor 975 kleintools.hms.harvard.edu/tools/currentDatasetsList_xenopus_v2.html. NNE = non-

976 neural ectodermal precursors; BC – basal cells; ISC = ionocytes; MCC = multiciliated

977 cells; SSC = small secretory cells; GB = 976 neural ectodermal precursors; BC – basal cells; ISC = ionocytes; MCC = multiciliated cells; SSC = small secretory cells; GB = outer-layer goblet cells. (C) Representative images of st. 9 control (ctrl.) and manipulate cells; SSC = small secretory cells; GB = outer-layer goblet cells. **(C)** Representative

images of st. 9 control (ctrl.) and manipulated embryos (animal views) after mRNA

overexpression of transcription factors to test p 978 images of st. 9 control (ctrl.) and manipulated embryos (animal views) after mRNA
979 overexpression of transcription factors to test premature induction of *dlc*. Quantification
980 of results and effects on *dll1* (y overexpression of transcription factors to test premature induction of *dlc*. Quantification

980 of results and effects on *dll1* (yellow) and *dlc* (blue) graphs. Embryos were scored as

981 induced or non-induced expres

of results and effects on *dll1* (yellow) and *dlc* (blue) graphs. Embryos were scored as

981 induced or non-induced expression. Related to Fig. 3A.

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 Figure S5: Effects of Notch manipulation of mucociliary deve 981 induced or non-induced expression. Related to Fig. 3A.

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 Figure S5: Effects of Notch manipulation of mucoci
 (A) IF of control (ctrl.), *dll1* (*dll1* MO) and *notch*

986 morphants after Notch gain of f 983
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989 984
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990 **Figure S5: Effects of Notch manipulation of mucociliary development**

985 **(A)** IF of control (ctrl.), $d\theta$ and $d\theta$ and $d\theta$ and $d\theta$ (notch and $d\theta$) momorphants after Notch gain of function (co-injected with *nic* 985 **(A)** IF of control (ctrl.), *dll1* (*dll1* MO) and *notch1* (*notch1* MO) morphants and morphants after Notch gain of function (co-injected with *nicd*) at st. 32 stained for Acetylated- α -tubulin (Ac.- α -tub., c 986 morphants after Notch gain of function (co-injected with *nicd*) at st. 32 stained for
987 Acetylated-α-tubulin (Ac.-α-tub., cilia, grey), F-actin (Actin, cell borders and morphology,
988 grey), and mucus (PNA, magen 987 Acetylated-α-tubulin (Ac.-α-tub., cilia, grey), F-actin (Actin, cell borders and morphology, grey), and mucus (PNA, magenta). Targeted cells were identified by membrane GFP expression (memGFP, green). Quantification 988 grey), and mucus (PNA, magenta). Targeted cells were identified by membrane GFP expression (memGFP, green). Quantification of cell type composition is depicted as pie-
990 charts, goblet cells (blue), ISCs (yellow), M 989 expression (memGFP, green). Quantification of cell type composition is depicted as pie-
990 charts, goblet cells (blue), ISCs (yellow), MCCs (green) and SSCs (red). n embryos
991 (above chart) and n quantified cells (990 charts, goblet cells (blue), ISCs (yellow), MCCs (green) and SSCs (red). n embryos (above chart) and n quantified cells (in/left of chart). **(B)** WMISH for cell type markers (*foxi1* = ISC; *foxi1* = MCCs; *foxa1* = S (above chart) and n quantified cells (in/left of chart). **(B)** WMISH for cell type markers $(foxi1 = \text{ISC}; foxi1 = \text{MCCs}; foxi1 = \text{SSCs}; \text{basal cells} = \Delta N-tp63)$ at st. 16/17 in controls and after knockdown of *dll1* (*dll1* MO). + = low do ($foxi1 = \text{ISC}$; $foxj1 = \text{MCCs}$; $foxi1 = \text{SSCs}$; basal cells = $\Delta N \text{-} tp63$) at st. 16/17 in controls
and after knockdown of $dl11$ ($dl11$ MO). $+$ = low dose, $++$ = medium dose, $+++$ = high
dose. Sections of embryos allow de 993 and after knockdown of $dll1$ ($dll1$ MO). $+$ = low dose, $++$ = medium dose, $++$ = high dose. Sections of embryos allow detailed comparison between injected ($dll1$ MO) and uninjected control (ctrl.) sides of embryos. G dose. Sections of embryos allow detailed comparison between injected (*dll1* MO) and

uninjected control (ctrl.) sides of embryos. Graphs: Quantification of results. Expression

levels were scored more, equal, less express 995 uninjected control (ctrl.) sides of embryos. Graphs: Quantification of results. Expression
996 levels were scored more, equal, less expression on the injected vs. uninjected sides. n
997 = number of embryos.
31 996 levels were scored more, equal, less expression on the injected vs. uninjected sides. n
997 = number of embryos.
31 997 = number of embryos.

997 = number of embryos.

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- Bowden, Brislinger-Engelhardt, Hansen et al. Foxi1 in mucociliary development
 Figure S6: Foxi1 is required for genomic accessibility of mucociliary genes
 (A-D) Distribution of accessible regions around genes requir 999
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005 Figure S6: Foxi1 is required for genomic accessibility of mucociliary genes
1001 (A-D) Distribution of accessible regions around genes required for development a
1002 fates specification in the embryonic mucociliary epider 1001 **(A-D)** Distribution of accessible regions around genes required for development and cell

1002 fates specification in the embryonic mucociliary epidermis of *Xenopus*. Lost, maintained

1003 and gained tracks as gen
-
- 1002 fates specification in the embryonic mucociliary epidermis of *Xenopus*. Lost, maintained

2003 and gained tracks as generated by MACS2 bdgdiff analysis and visualized in IGV. (A)

2004 *ubp1.L*; (B) *dmrt2.S*; (C) 1003 and gained tracks as generated by MACS2 bdgdiff analysis and visualized in IGV. **(A)** $ubp1.L$; **(B)** $dmrt2.S$; **(C)** $foxj1.L$; and **(D)** $tp63.L$. Turquoise track = control (ctrl.) and purple track = morphant *(foxi1 MO)*.
- 1004 *ubp1.L;* **(B)** *dmrt2.S;* **(C)** *foxj1.L;* and **(D)** *tp63.L.* Turquoise track = control (ctrl.) and purple track = morphant (foxi1 MO). n = 2 organoids per condition and replicate. 3 replicates.
- 1005 purple track = morphant (*foxi1* MO). n = 2 organoids per condition and replicate. 3
1006 replicates.
- 1006 replicates.

1006 replicates.

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Ac.-a-tub. + Actin + Serotonin / targeted (H2B-RFP) / foxi1::GFP-Utrophin

.-a-tub. + Actin + Serotonin / targeted (H2B-RFP) / foxi1∆1::GFP-Utrophin Ac

foxi1::GFP-Utrophin

foxi1∆1::GFP-Utrophin

100

gfp-intensity (AU)

foxi1::GFP-Utrophin

B

targeted (memRFP) / foxi1::GFP-Utrophin

memRFP 100 gfp-intensity (AU) foxi1 + memRFP

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