FGF signalling regulates enhancer activation during ear progenitor induction

Monica Tambalo^{\$}, Maryam Anwar[#], Mohi Ahmed and Andrea Streit^{*}

Centre for Craniofacial and Regenerative Biology, Faculty of Dental, Oral and Craniofacial Sciences, King's College London, London SE1 9RT, UK

^{\$} current address: The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK

[#] current address: Imperial College London, Institute of Clinical Sciences, Faculty of Medicine, South Kensington Campus, London, SW7 2AZ, UK

^{*} corresponding author: andrea.streit@kcl.ac.uk

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1 **ABSTRACT**

2
3 The fibroblast growth factor pathway is essential for inner ear induction in many
4 vertebrates, however how it regulates the chromatin landscape to coordinate the
5 activation of otic genes remains unclear. Here we show that FGF exposure of
6 sensory progenitors leads to rapid deposition of active chromatin marks H3K27ac
7 near hundreds of FGF-responsive, otic-epibranchial progenitor (OEP) genes, while
8 H3K27ac is depleted in the vicinity of non-otic genes. Genomic regions that gain
9 H3K27ac act as cis-regulatory elements controlling OEP gene expression in time and
10 space and define a unique transcription factor signature likely to control their activity.
11 Finally, we provide evidence that in response to FGF signalling the transcription
12 factor dimer AP1 recruits the histone acetyl transferase p300 to OEP enhancers and
13 that de novo acetylation is required for subsequent expression of OEP genes. Thus,
14 during ear induction FGF signalling modifies the chromatin landscape to promote
15 enhancer activation and chromatin accessibility.

INTRODUCTION

The vertebrate inner ear is critical to relay auditory and vestibular information from the environment to the brain. Developmental malformations or postnatal damage of inner ear cells lead to permanent sensory defects as the ear does not have the ability to regenerate or repair. Despite extensive studies, our understanding of normal ear development has not yet translated into new biological approaches to alleviate such disorders or to promote sensory cell regeneration *in vivo*. This is partly due to the lack of mechanistic information downstream of signalling pathways that control ear development and of how signalling events are translated into changes in gene expression and cell behaviour.

The Fibroblast Growth Factor (FGF) pathway is crucially important for many steps in ear formation. During early development, FGF signalling mediates the induction of otic-epibranchial progenitors (OEPs) from a pool of sensory stem cells (Ladher et al., 2000; Maroon et al., 2002; Park and Saint-Jeannet, 2008; Phillips et al., 2001; Sun et al., 2007; Urness et al., 2010; Wright and Mansour, 2003; Yang et al., 2013a). Otic precursors segregate from epibranchial progenitors to form a transient structure, the otic placode, and subsequently the otic vesicle, which gives rise to the entire inner ear including the cochlear-vestibular ganglion (Chen and Streit, 2013; Groves and Fekete, 2012; Ladher, 2017; Ohyama et al., 2007; Whitfield, 2015). During these stages, FGF signalling promotes otic neurogenesis and mediates patterning events that subdivide the otic vesicle into distinct functional domains (Abello et al., 2010; Adamska et al., 2001; Alsina et al., 2004; Alvarez et al., 2003; Hammond and Whitfield, 2011; Leger and Brand, 2002; Maier and Whitfield, 2014). As the vesicle gradually acquires the shape of the adult ear, sensory patches emerge that generate auditory and vestibular sensory hair cells and their surrounding supporting cells. Within the cochlea, these cells form a stereotypical pattern that is critical for normal hearing, with FGF signalling controlling both their arrangement and their differentiation (Doetzlhofer et al., 2009; Haque et al., 2016; Mueller et al., 2002; Shim et al., 2005). Finally, FGFs have also been implicated in hair cell survival in adult ears as well as controlling their regeneration from supporting cells (Jiang et al., 2018; Kniss et al., 2016; Lee et al., 2016; Lush et al., 2019).

Upon ligand binding FGF receptors initiate different intracellular cascades including the ERK/MAPK pathway (Ornitz and Itoh, 2015) to regulate gene expression and subsequent changes in cell behaviour. Signalling information is integrated by non-coding regulatory enhancer regions to activate or repress downstream targets (Banerji et al., 1981; Catarino and Stark, 2018; Long et al., 2016;

1 Shlyueva et al., 2014). Active enhancers are characterised by low nucleosome
2 density (Boyle et al., 2008), recruitment of the histone acetylase p300 (Visel et al.,
3 2009), acetylation of histone 3 lysine-27 (H3K27ac) in the enhancer-flanking
4 nucleosomes (Creyghton et al., 2010; Kharchenko et al., 2011; Rada-Iglesias et al.,
5 2012; Rada-Iglesias et al., 2011; Zentner et al., 2011), and the expression of RNA
6 transcripts (Kim et al., 2010; Wang et al., 2011). Downstream of FGF signalling,
7 different mechanisms regulate target gene expression. On one hand, Erk1/2 MAP-
8 kinases can directly modify nucleosome forming histones and change epigenetic
9 marks (Chen et al., 2007; Soloaga et al., 2003), and their inhibition can increase
10 chromatin accessibility (Patel et al., 2013; Semprich et al., 2019). On the other hand,
11 MAP kinases phosphorylate Ets transcription factors (e.g. Ets4 and Ets5) or the
12 dimer forming leucine zippers cFos and cJun (AP1) (Gruda et al., 1994; Neuberger et
13 al., 1989; Ornitz and Itoh, 2015; Tsang and Dawid, 2004) (for review: (Yang et al.,
14 2003, 2013b), which then bind to enhancers and together with other factors, activate
15 target genes. For example, AP1 cooperates with cell type specific transcription
16 factors to recruit the BAF complex, which in turn remodels nucleosomes to establish
17 a permissive chromatin state (Vierbuchen et al., 2017). Thus, modulation of FGF
18 signalling appears to control changes in the epigenetic signature. However, in the
19 context of *in vivo* ear development, the molecular mechanisms that translate FGF
20 signalling into rapid transcriptional changes remain to be elucidated.

21 Here we study chromatin changes in response to FGF signalling during the
22 earliest step of ear development, the induction of otic-epibranchial progenitors. We
23 find that FGF stimulation of sensory precursors leads to deposition of H3K27ac
24 marks in the vicinity of ear-specific, FGF-response genes and that these genomic
25 regions act as ear-specific enhancers. *De novo* acetylation of H3K27 is required for
26 otic gene expression and fate allocation. We show that AP1 plays a key role in this
27 process: upon FGF signalling, AP1 recruits the histone acetylase p300 to ear
28 enhancers, which in turn promotes H3K27 acetylation associated with increased
29 chromatin accessibility and enhancer activation. Together these findings highlight
30 that during ear induction, the initial response to Erk/MAPK signalling directly
31 activates ear-specific enhancers, providing a molecular mechanism for rapid
32 activation of gene expression downstream of FGF. In turn, these observations may
33 impact on a variety of diseases and developmental disorders where FGFs play a
34 major role.

1 RESULTS

2 FGF signalling induces dynamic changes in H3K27 acetylation

3 FGF signalling is critical to initiate the ear programme. Loss of FGFs or pathway
4 inhibition results in the complete absence of ear precursors, while exposure of
5 sensory progenitors to FGF induces otic epibranchial progenitors (OEPs) (Ladher et
6 al., 2000; Maroon et al., 2002; Park and Saint-Jeannet, 2008; Phillips et al., 2001;
7 Sun et al., 2007; Urness et al., 2010; Wright and Mansour, 2003; Yang et al., 2013a).
8 Only few FGF targets have been identified, and the downstream epigenetic changes
9 are unknown (Anwar et al., 2017; Yang et al., 2013a). To define the initial events as
10 sensory progenitor cells are induced to become OEPs, we assessed changes in
11 Histone 2 lysine 27 acetylation (H3K27ac) and trimethylation (H3K27me3) in
12 response to FGF signalling. Histone acetylation is associated with increased
13 accessibility of chromatin and gene activation, marking active enhancers and
14 promoters, while H3K27me3 is associated with gene repression (for review see:
15 Maston et al., 2012, Calo and Wysocka, 2013, Kimura, 2013). Sensory progenitors
16 from HH6 chick embryos were cultured in the presence or absence of FGF2 for 6 hrs
17 and processed for chromatin immunoprecipitation for H3K27ac and H3K27me3
18 followed by sequencing (nano-ChIP-seq; Figure 1A; (Adli and Bernstein, 2011). In
19 both control and FGF-treated cells, transcription start sites (TSS) show H3K27ac
20 accumulation and H3K27me3 depletion (Figure 1B). However, genome-wide
21 comparison of both samples reveals significant changes in the distribution of
22 H3K27ac marks.

23 Association of H3K27ac peaks to the nearest TSS reveals that genes
24 normally expressed in OEPs like the direct FGF targets *Spry1* and *Spry2*, the FGF-
25 responsive transcript *Foxi3*, the transcription factor *Hesx1* and the chemokine *Cxcl14*
26 show a marked gain in H3K27ac (Figure 2A, D; Supplementary Figure 1;
27 Supplementary Figure 2A). In contrast, transcripts normally absent from OEPs and
28 repressed by FGF signalling like *Pax6*, *Dlx5/6* and *Gata3* show a significant loss
29 (Figure 2G; Supplementary Figure 6B-C), while there is no change in H3K27ac near
30 sensory progenitor genes like *Eya2* (Figure 2H), whose expression precedes OEP
31 induction by FGF (Ahrens and Schlosser, 2005; Litsiou et al., 2005; Pandur and
32 Moody, 2000; Sato et al., 2010). Thus, changes in histone acetylation reflect
33 changes in gene expression as sensory progenitors are specified as otic-epibranchial
34 progenitors.

1 H3K27ac marks FGF-responsive enhancers in OEPs

2 Activation of gene expression is controlled by cis-regulatory elements and active
3 enhancers are normally flanked by H3K27ac peaks (Figure 1C) (for review see:
4 Maston et al., 2012, Calo and Wysocka, 2013, Kimura, 2013). Therefore, the
5 increase in H3K27ac upon FGF stimulation may indicate the activation of ear
6 enhancers. To identify putative enhancers genome-wide, we extracted bimodal
7 H3K27ac peaks with a maximum distance of 3kb (Figure 1D) in sensory progenitors
8 (control) and FGF-induced OEPs; these are mostly located in intronic or intergenic
9 regions (Figure 1E). We find 2451 putative enhancers that are unique to control cells,
10 2883 specific to FGF2-treated progenitors, and 808 are common to both (Figure 1F,
11 Supplementary Figure 3). FGF-induced putative enhancers preferentially associate
12 with genes enriched in otic progenitors (from Chen et al., 2017): comparing all OEP
13 transcripts to those with putative active enhancers shows significantly higher
14 expression levels of the latter (Supplementary Figure 4). In addition, GO term
15 analysis confirms that putative enhancer associated genes are linked to terms like
16 inner ear development and MAP kinase signalling, while these terms are absent from
17 genes in controls (Figure 1G).

18 To assess whether the genomic regions flanked by H3K27ac are indeed
19 active OEP enhancers *in vivo*, we selected elements associated to known FGF
20 targets or OEP genes, cloned them into reporter constructs to drive eGFP and
21 assessed their activity in chick embryos. HH6 embryos were electroporated with
22 enhancer-eGFP vectors together with a control vector driving RFP ubiquitously.
23 There are four putative enhancers near *Spry1*, of which two were tested for *in vivo*
24 activity. *Spry1*-E2 is conserved across vertebrate species as determined by DREiVE
25 (Khan et al., 2012) (Figure 2A) and drives eGFP in otic progenitors from the 2-somite
26 stage (ss) onwards (Figure 2B, C) recapitulating the normal expression pattern of
27 *Spry1* (compare to Figure 4D), while E1 shows no activity (not shown). Two
28 evolutionary conserved elements are associated with *Foxi3* (*Foxi3*-E1 and -E2;
29 Figure 2D), a transcription factor crucial for ear development (Birol et al., 2016; Khatri
30 et al., 2014; Solomon et al., 2003); both drive reporter expression in two spatially and
31 temporally distinct domains (Figure 2E, F; Supplementary Figures 5, 6). *Foxi3*-E1 is
32 active early in OEPs, but is shut down after the 5-6ss, while *Foxi3*-E2 becomes
33 active slightly later, when *Foxi3* is lost from the otic placode, and is confined to non-
34 otic ectoderm. Thus, both enhancers recapitulate the dynamic expression of *Foxi3* in
35 the head ectoderm (Khatri et al., 2014). In addition, enhancers associated with the
36 otic transcription factor *Hesx1* and with the cytokine *Cxcl14* are also active in the otic

territory (Supplementary Figure 6). Therefore, H3K27ac enrichment in response to FGF signalling identifies ear-specific enhancer regions.

Unique transcription factor motifs define OEP enhancers

Enhancers serve as transcription factor hubs that integrate different transcriptional inputs to activate cell fate specific gene expression. To investigate whether a unique transcriptional signature might regulate OEP genes, we performed motif enrichment. Using RSAT matrix-scan we scanned for TF binding sites enriched in FGF2-treated versus control cells and vice versa using JASPAR and TRANSFAC libraries to identify known motifs. TFAP2 motifs (Figure 3A; Supplementary File1 - Cnt_M14_M17_M26) and motifs where the corresponding transcription factors are currently unknown (Supplementary File1) are highly overrepresented in control enhancers. In addition, they harbour *Zic1/3*, *Srebf2* and *Klf5* binding sites and the corresponding transcription factors *TFAP2a*, *-b*, *-c* and *-e*, *Klf5* and *Zic1/3* are expressed in sensory progenitors, while *Srebf2* seems to be expressed ubiquitously (Figure 3C; Supplementary Figure 7). Indeed, TFAP family members expressed in neural plate border cells control both neural crest and sensory progenitor fates (Hoffman, 2007; Knight et al., 2003; Li and Cornell, 2007; Qiao et al., 2012), while *Zic* transcription factors together with *Pax3* discriminate between both fates (Hong and Saint-Jeannet, 2007).

In contrast, OEP associated enhancers show a different motif signature. Motifs corresponding to homeobox and winged-helix/forkhead domain transcription factors are highly enriched (Supplementary File2 FGF2_M5_12_15_21_16_20_29), as are binding sites for basic-loop-helix factors (Supplementary File2 FGF2_M8), for the transcription factor *Myb* (Supplementary File2 FGF2_M7) and the FGF signalling mediators of the Jun and Fos families (Figure 3B; Supplementary File2 FGF2_M18). Many of the corresponding transcription factors are expressed in OEPs and required for their specification including *Dlx5/6/3*, *Gbx2*, *Lmx1a* and *Six1* and the forkhead factor *Foxi3* (Birol et al., 2016; Brugmann et al., 2004; Chen et al., 2017; Christophorou et al., 2009; Kaji and Artinger, 2004; Khatri et al., 2014; Luo et al., 2001; McLarren et al., 2003; Sato et al., 2010), while others play a role at later stages of ear development (e.g. *Foxg1*) (Hwang et al., 2009) and yet others are expressed in OEPs, but their function has not been investigated (*FoxP1*, *FoxC2*, *FoxK2*; (Chen et al., 2017). Thus, a unique signature of transcription factor binding sites defines OEP enhancers and identifies new potential regulators of OEP identity.

The Fos/Jun transcription factors are mediators of FGF signalling and form homo- or heterodimers (AP1) to activate downstream targets. The AP1 motif was

1 differentially associated with FGF2-induced elements (Figure 3B; Supplementary
2 File1 – FGF2_M18) and is indeed present in the enhancers tested *in vivo* (Figure 2A,
3 D; Supplementary Figure 5C). This finding suggests a major difference in enhancer
4 activation during OEP induction can be attributed to the FGF pathway. Examining the
5 distribution of H3K27Ac within 5kb surrounding AP1 motifs reveals a strong
6 correlation in FGF-treated cells, while the same regions are depleted in controls
7 (Figure 3E, F). Clustering and peak density plots identifies five different clusters (C1-
8 C5) that differ in the location of H3K27ac marks with respect to AP1 motifs (Figure
9 3F). Functional annotation of these clusters shows enrichment of terms like ear
10 morphogenesis, inner ear development, sense organ development and, importantly,
11 MAPK signalling (Supplementary Figure 8). Together, these data define a unique set
12 of transcription factor binding sites over-represented in FGF activated enhancers
13 when compared to control enhancers. The corresponding transcription factors are
14 known to control otic identity, while others represent new putative upstream
15 regulators.

16 **Genes associated to FGF-activated enhancers are FGF targets in OEPs**

17 The above experiments identified several thousand enhancers that are rapidly
18 activated in response to FGF signalling. However, previous studies identified only
19 few immediate FGF targets (Anwar et al., 2017) or few genes induced after 24hrs'
20 exposure to FGF (Yang et al., 2013a). We therefore investigated whether genes
21 associated with FGF-activated enhancers are indeed regulated by this pathway. We
22 have recently characterised the transcriptional profile of OEPs (Chen et al., 2017)
23 and based on these data designed a NanoString probe set with 216 genes
24 containing 70 ear specific factors, as well as transcripts normally expressed in
25 progenitors for other sense organs, cranial ganglia, neural and neural crest cells.
26 Sensory progenitors from HH6 chick embryos were cultured in the absence or
27 presence of FGF2 for 3 and 6 hours and changes in gene expression were evaluated
28 by NanoString (Figure 4A). After 3 hrs known FGF targets (*Etv4/5*, *Spry1/2*) are
29 strongly up-regulated together with early OEP markers (e.g. *Foxi3*, *Gbx2*, *Pax2*,
30 *Sox13* and *Klf7*; in total 16 otic TFs), while genes normally expressed in other cell
31 types (e.g. *Pax6*, *Otx2*, *Msx1*, *Id2*, *-4*) and some late otic genes (*Sall4*, *Zfhx3*, *Fez1*,
32 *Lmx1b*, *Myb*) are repressed (Figure 4B, D; Supplementary File 3). After 6 hrs of FGF
33 exposure, the expression of most early induced transcripts is maintained and a few
34 new factors become upregulated (e.g. *Eya2*, *N-Myc*, *Tcf7L2*; Figure 4C;
35 Supplementary File 4). These changes in gene expression correlate well with

enhancer activation by FGF signalling: most upregulated transcripts are associated with an FGF-induced gain of H3K27ac (Supplementary Figure 3, 4). Together, these experiments suggest that FGF signalling initiates OEP induction by directly activating enhancers of ear specific genes, and that this activity maybe mediated by AP1 together with other transcription factors.

AP1 recruits the histone acetyl-transferase p300 to ear-specific enhancers

What is the mechanism by which FGF and AP1 activate OEP enhancer regions? Acetylation of H3K27 is mediated by histone acetyl-transferases (HAT) like p300. To assess whether p300 occupies otic enhancers, we selected two experimentally verified enhancers, *Spry1-E1* and *Foxi3-E1*, with a significant gain of H3K27ac in response to FGF activation. We electroporated p300-Flag into ear progenitors and performed anti-Flag ChIP-qPCR from targeted tissue. Indeed, p300 is bound to both enhancers, but is absent from the enhancer of the muscle gene *MyoD* (Figure 5B). To test whether *de novo* acetylation is required for OEP gene expression we treated sensory progenitors with FGF2 in the presence or absence of the p300 inhibitor C646 (Bowers et al., 2010; Zhang et al., 2013) and assessed OEP markers after 3 hrs by qRT-PCR. When p300 is inhibited the expression of the FGF-dependent OEP transcripts *Pax2* and *Cxcl14* is no longer induced. In addition, there is a non-significant reduction of *Spry1*, *Gbx2*, *Foxi3* and *Sox13*, while *Six1* does not change in line with its FGF independence (Figure 5A). Using the general HAT inhibitor anacardic acid has the same effect (data not shown). Therefore, *de novo* acetylation of p300 targets is required for OEP gene expression.

AP1 is known to interact with p300 (Crish and Eckert, 2008), raising the possibility that AP1 recruits p300 to OEP enhancers to facilitate histone acetylation. To test this, we made use of a dominant negative form of Fos (acidic-Fos: a-Fos; (Biddie et al., 2011), a truncated version of Fos that maintains its interaction with Jun, but cannot bind DNA. HH6 chick embryos were electroporated with p300-Flag together with a-Fos or RFP (control) constructs; targeted otic progenitors were dissected and processed for ChIP-qPCR using anti-flag antibodies. While p300 binding to *Spry1-E1* and *Foxi3-E1* is observed in controls, this is inhibited in the presence of a-Fos (Figure 5B). No binding is observed at the *MyoD* enhancer, a transcript not expressed in ear progenitors. These findings suggest that in response to FGF signalling, AP1 recruits the HAT p300, which in turn increases acetylation of

- 1 H3K27 at ear-specific enhancers and as a result rapidly induces their expression
- 2 (Figure 5C).
- 3

1 DISCUSSION

2 **FGF signalling mediates enhancer activation in otic-epibranchial progenitors**

3 In the embryo, cell fate choices are mediated by temporally and spatially controlled
4 signals that activate distinct transcriptional programmes in responding cells. The key
5 elements that integrate this information are non-coding, cis-regulatory regions that
6 control cell type specific gene expression, which in turn implement fate decisions
7 (Banerji et al., 1981; Beagrie and Pombo, 2016; Pennacchio et al., 2013). Enhancer
8 elements are characterised by specific epigenetic signatures, however how transient
9 signalling cues during development affect the chromatin landscape is not well
10 understood. Active enhancers are nucleosome-free regions flanked by characteristic
11 histone modifications including acetylation of histone 3 at lysine 27 (Creyghton et al.,
12 2010; Kharchenko et al., 2011; Rada-Iglesias et al., 2012; Rada-Iglesias et al., 2011;
13 Zentner et al., 2011). Here we study the changes in H3K27ac as sensory progenitors
14 acquire OEP fate in response to FGF signalling. We find that FGF rapidly induces
15 dynamic changes at thousands of genomic regions with a marked gain of H3K27
16 acetylation near OEP genes. We show that these act as enhancer elements that
17 drive OEP-specific gene expression *in vivo* and enhancer associated transcripts are
18 indeed rapidly upregulated by FGF. In contrast, genomic regions surrounding genes
19 normally absent in OEPs lose H3K27 acetylation, and these transcripts are
20 repressed by FGF. Thus, we have identified thousands of FGF-activated and -
21 repressed regulatory elements during OEP induction providing a rich resource to
22 explore the gene regulatory network that controls ear development.

23

24 **AP1 mediates p300 recruitment to FGF activated enhancers**

25 FGF signalling activates gene expression in responding cells within minutes, and our
26 studies provide a molecular mechanism underlying this response. In ear progenitors,
27 FGF is mediated by the MAP-kinase pathway (Yang et al., 2013a) leading to
28 phosphorylation of the transcription factors Jun and Fos, which work as a
29 heterodimer AP1 to activate gene expression (Ornitz and Itoh, 2015; Tsang and
30 Dawid, 2004; Yang et al., 2003, 2013b). The AP1 binding motif is strongly enriched in
31 FGF-activated enhancers, and we show that AP1 is required to recruit the histone
32 acetyltransferase p300 to ear enhancers. Indeed, AP1 and p300 physically interact
33 (Crish and Eckert, 2008), and inhibition of p300 prevents induction of FGF response
34 genes in sensory progenitors. This supports the idea that p300 activity and
35 subsequent increased histone acetylation is required to activate OEP genes. A

1 similar mechanism has recently been reported in HeLa cells (O'Donnell et al., 2008).
 2 Here, in response to MAP kinase signalling, Elk is phosphorylated and together with
 3 p300 changes the histone acetylation state, which in turn allows the recruitment of
 4 other transcription factors and subsequent gene activation. It is therefore possible
 5 that increased acetylation in OEPs enhances chromatin accessibility allowing other
 6 otic transcription factors to access target enhancers, where they may cooperate with
 7 AP1 to activate target transcription. Motif enrichment analysis of FGF-induced ear
 8 enhancers suggests that homeobox and winged-helix/forkhead proteins are possible
 9 cooperators. Of those, the homeobox genes *Gbx2*, *Six1* and *Lmx1a* and the forkhead
 10 gene *Foxi3* are indeed expressed in OEPs and are required for their specification
 11 and/or vesicle formation (Birol et al., 2016; Brugmann et al., 2004; Chen et al., 2017;
 12 Christophorou et al., 2009; Kaji and Artinger, 2004; Khatri et al., 2014; Luo et al.,
 13 2001; McLarren et al., 2003; Sato et al., 2010). In addition, we have recently shown
 14 that Myb recruits the histone demethylase Lsd1 to OEP gene promoters to maintain
 15 their expression after initial specification (Ahmed and Streit, 2018), while our current
 16 results suggest that Myb may also cooperate with AP1 at OEP enhancer regions.

17 In addition, AP1 is known to influence chromatin accessibility using different
 18 mechanisms and may act as a pioneer factor to mediate chromatin opening. In
 19 mouse embryonic fibroblasts, AP1 can bind to nucleosome-occupied regulatory
 20 elements together with cell type specific transcription factors (Vierbuchen et al.,
 21 2017). Together, they recruit the SWI/SNF complex BAF, which repositions
 22 nucleosomes to increase enhancer accessibility. Likewise, AP1 is required for
 23 glucocorticoid induced transcription by priming glucocorticoid receptor target sites
 24 and maintaining their accessibility before the receptor can be recruited (Biddie et al.,
 25 2011). Whether similar mechanisms also operate in OEPs in response to FGF
 26 signalling remains to be uncovered.

27 FGF signalling not only promotes OEP fate, but also shuts down alternative
 28 differentiation programmes. For example, we have previously shown that the lens
 29 programme is inhibited in OEPs by FGFs with the lens marker *Pax6* being a direct
 30 target (Anwar et al., 2017; Bailey et al., 2006). Here we observe a significant
 31 decrease of H3K27ac in the *Pax6* locus and a complementary increase of
 32 H3K27me3. Recent evidence suggests that AP1 may mediate transcriptional
 33 repression by recruiting histone deacetylases (Miotto et al., 2006; Mittelstadt and
 34 Patel, 2012), which subsequently allows H3K27 methylation. Interestingly, in the
 35 neural tube FGF signalling promotes compaction of the *Pax6* locus mediated by
 36 polycomb repressive complexes (Patel et al., 2013; Semprich et al., 2019), while
 37 inhibition of Erk1/2 results in dissociation of this complex from the *Pax6* TSS. It is

1 therefore possible that in OEPs increased H3K27me3 deposition at the Pax6 locus
2 allows recruitment of the polycomb repressor complex to repress *Pax6*, a key gene
3 for lens formation. Thus, AP1 function depends on a fine balance between activator
4 and repressor complexes, presumably controlled by the availability of different,
5 enhancer specific co-factors.

6 **OEP induction: new transcription factors downstream of FGF signalling**

7 It is well established that FGF signalling activates the inner ear programme by
8 inducing otic-epibranchial progenitors from a pool of multipotent precursors. While
9 continued FGF activation promotes epibranchial identity, Notch together with Wnt
10 signalling establishes the otic placode (Freter et al., 2008; Ohyama et al., 2006) (for
11 review (Chen and Streit, 2013; Groves and Fekete, 2012; Ladher, 2017; Ohyama et
12 al., 2007; Whitfield, 2015). Here we identify new FGF-response genes and their
13 associated enhancers. Using network inference approaches we have recently
14 proposed that FGFs initially enhances the transcription of genes already expressed
15 in sensory progenitors, which then act in a positive feedback loop to increase their
16 own expression (Anwar et al., 2017). Downstream of this circuit, *Pax2* and other
17 transcription factors are activated to regulate OEP identity while repressing
18 alternative fates. Here, we show that within 3 hrs FGF leads to the activation of 20
19 transcription factors, many of which are expressed in OEPs, but absent from other
20 placodes. These include the known targets *Etv4* and -5 and the FGF antagonists
21 *Spry-1* and -2, as well as many new FGF-responsive factors like *Sox13*, *Tead3* and
22 *Klf7*. Transcription factor binding motifs for these factors are present in ear
23 enhancers suggesting that they may also play a key role in the genetic programme
24 controlling early ear induction. Indeed, in *Xenopus* and mouse, the transcription
25 factor *Klf7* is expressed in the otic placode (Gao et al., 2015; Laub et al., 2001). *Klf7*
26 has been implicated in stem cell renewal, as well as cell cycle exit of neuronal
27 precursors (Jeon et al., 2016; Kajimura et al., 2007; Laub et al., 2006; Laub et al.,
28 2005), however its function in ear development remains unknown.

29
30 In summary, during the induction of otic-epibranchial progenitors, FGF signalling
31 rapidly activates downstream target genes by increasing H3K27 acetylation at
32 enhancer regions. FGF activity is mediated by the Jun/Fos heterodimer AP1, which
33 recruits the acetyltransferase p300 to OEP enhancers. The genome-wide
34 identification of FGF-responsive OEP enhancer regions forms the basis to

- 1 reconstruct the otic induction network and may shed light on other FGF-mediated
- 2 processes in the ear.
- 3

METHODS

Embryo manipulation and explant culture

Experiments on chick embryos prior to E10 do not require a home office license or institutional approval and were carried out according to the institutional guidelines. Fertilized chicken eggs were obtained from Winter Farm (Herts, UK) and incubated in a humidified incubator at 38°C until reaching the desired stage (Hamburger and Hamilton, 1951). For electroporation, embryos were cultured using the filter culture method (Chapman et al., 2001). To isolate sensory progenitor explants, embryos were collected in Tyrode's saline and progenitors were dissected using fine needles, and cultured in DMEM supplemented with FGF2 (0.25ng/μl; R&D), DMSO or C646 (10μM; Calbiochem) for 3 and 6 hours.

Enhancer cloning and electroporation

Putative enhancer elements were amplified from chick genomic DNA and inserted into pTK vector containing eGFP reporter and a TK minimal promoter (Kondoh and Uchikawa, 2008). Cloning primers are listed in Supplementary Table1. Flagged tagged p300 vector was kindly provided by Michael O. Hottiger (Hasan et al., 2001). For electroporation, embryos on filter paper were transferred into an electroporation chamber containing a 2x2mm platinum electrode. DNA (3 μg/μl reporter DNA pTK eGFP and 1.5 μg/μl pCAB RFP, 0.1% fast green; 1 μg/μl p300-Flag and 1.5 μg/μl pCAB RFP, 0.1% fast green) was injected by air pressure between the vitelline membrane and embryonic ectoderm, a platinum electrode (2x1 mm) was placed above the target area and 5 pulses of 4V for 50ms with 750ms intervals were applied using Intracel TSS20 OVODYNE pulse generator. Embryos were cultured overnight and imaged using an inverted fluorescent microscope.

Quantitative RT-PCR and NanoString nCounter analysis

RNA from cultured sensory progenitor explants treated with FGF2 (0.25ng/μl; R&D), DMSO or C646 (10μM; Calbiochem) for 3 hrs was isolated using the RNAqueous-Micro Kit (Ambion) and reverse transcribed. Primers for target genes were designed with PrimerQuest (IDT). qPCR was performed in technical triplicates using Rotor-Gene Q (Qiagen) with SYBR green master mix (Roche). The $\Delta\Delta C_t$ method was used to calculate the fold change, which was expressed as $FC=2^{-(\Delta\Delta C_t)}$ (Livak and Schmittgen, 2001). Gapdh and Sdha were used as reference genes to calculate the fold change. Two biological replicates were performed and the P-value generated from a two-tailed Student's t-test was used to evaluate statistical significance.

For Nanostring nCounter analysis, eight to ten explants per condition were lysed in 5µl lysis buffer (Ambion) and analyzed by nCounter® Analysis System (Life Sciences) using a customized probe set of 216 genes (Chen et al., 2017). Total RNA was hybridized with capture and reporter probes at 65 °C overnight. Target/probe complexes were washed and immobilized according to the nCounter Gene Expression Assay Manual, and data were collected by the nCounter Digital Analyzer. Experiments were repeated on three independent occasions and data were analyzed following company instructions. A cut-off of fold change ≥ 1.25 and ≤ 0.75 was used to identify upregulated and downregulated genes, respectively, in combination with a p-value ≤ 0.05 (unpaired t-test).

***In situ* hybridization**

The following chick plasmids were used to generate Digoxigenin–labeled antisense probes: Etv4 (a kind gift from M. Bronner) Spry1 obtained from Life Technologies Klf7 ChEST376015; Sox13 ChEST437d11; Pax2 (a kind gift from M. Golding) and Pax6 (a kind gift from A. Bang). RNA probes were synthesized with T7, T3 or SP6 RNA polymerase (Roche). Whole mount or explant *in situ* hybridization was performed as described previously (Streit and Stern, 2001).

Chromatin immunoprecipitation (ChIP)

Around 100 posterior sensory progenitors from HH6 were cultured for 6 hours in the absence or presence of FGF2. Explants were dissociated in 0.5ml of Nuclei Extraction Buffer (NEB; 0.5% NP-40, 0.25% Triton X-100, 10mM Tris-HCl pH 7.5, 3mM CaCl₂, 0.25M Sucrose, 1mM DTT, 0.2mM PMSF, 1X Protease Inhibitor) by homogenisation and crosslinked using 1% formaldehyde for 9 min at room temperature. 0.125M glycine final concentration was used to stop fixation. Samples were pelleted and stored at -80°C. 100µl of Dynal magnetic beads (Protein A, Novex Life Technologies) were incubated with 1ml of blocking buffer (1X PBS with 0.5% BSA) for 2 min, resuspended in 250µl of blocking buffer and respective antibodies were added (1µg/µl Rabbit anti-IgG Millipore – CA92590; 1µg/µl Rabbit anti-H3K27ac Abcam – ab4729; 2.5µg Rabbit anti-H3K27me3, Cell signaling – C36B11; 3µg anti-Flag, Sigma – F3165). Antibody and beads were incubated overnight rotating at 4°C, and excess of antibody was removed by washes in blocking buffer. Crossed-linked pellets were defrosted, washed in Nuclei Extraction Buffer and nuclei were extracted by homogenisation. Samples were lysed in 1% SDS, 50mM Tris-HCl (pH 8), 10mM EDTA supplemented with 20µl Protease Inhibitor (7x PI; Roche) for 1 hour. Prior to

1 sonication 280µl of ChIP dilution buffer (0.01% SDS; 16.7mM Tris-HCl pH 8; 1.2mM
2 EDTA; 167mM NaCl; supplemented with PMSF, DTT and PI) was added, samples
3 were sonicated to generate fragments of 200-600bp using 12 cycles of 15sec on and
4 30sec off at 40% amplitude (SONICS, Vibra Cell™). The resulting chromatin was
5 diluted 4x and divided across different samples, 1/10th of sonicated chromatin was
6 kept frozen as input control. Beads were washed 9 times in RIPA buffer containing
7 50mM Hepes-KOH (pH 8), 500mM LiCl, 1mM EDTA, 1% NP-40, 1.7% Na-
8 deoxycholate supplemented with PI. A final wash was performed using 1x TE, 50mM
9 NaCl and the chromatin was eluted in 50mM Tris-HCl (pH 8), 10mM EDTA and 1%
10 SDS. Reverse-crosslinking was carried out at 65°C overnight. Chromatin was diluted
11 using 1xTE and incubated at 37°C for one hour with RNase A 0.4mg/µl, followed by
12 proteinase K digestion for one hour at 55°C. Chromatin was purified using phenol:
13 chloroform: isoamyl alcohol and resuspended in 60µl of water. ChIPed chromatin
14 was processed for next generation sequencing or analyzed by qPCR. For qPCR Ct
15 values for each precipitation were extracted and normalized to input chromatin,
16 primer sequences are listed in Supplementary Table 2. For next generation
17 sequencing chromatin was amplified with a step of linear amplification following the
18 protocol described in (Adli and Bernstein, 2011) Library preparation was performed
19 at the UCL Genomics, Institute of Child Health following the protocol used for nano-
20 ChIP-seq (Adli and Bernstein, 2011) with the exception that only 12 cycles were used
21 in the PCR amplification.

22

23 **ChIP-seq data analysis**

24 Sequence quality was assessed using FastQC (Andrews, 2010). During each ChIP-
25 seq experiment, an amplification step was carried out that is reported to produce
26 mismatches at the first 9bp due to random priming (Adli and Bernstein, 2011).
27 Therefore, as the sequence quality was poor these 9bp were trimmed. Further
28 trimming was performed to improve alignment if sequence quality from the 3' end
29 was poor. Reads were aligned to the chick genome Galgal4.71 using Novoalign
30 (Novocraft 2.08.01, <http://www.novocraft.com/products/novoalign/>) and uniquely
31 aligned sequences were used for peak calling using Homer (Heinz et al., 2010) and
32 MACS2 (Zhang et al., 2008). For Homer, a fold change of 1.5 relative to input and a
33 False Discovery Rate (FDR) of 0.01 were used. For MACS2, a FDR of 0.05 as
34 suggested in the MACS manual to obtain broad peaks (characteristic of histone
35 peaks) and a default p-value of 1e-5 were used. Genome wide analysis was
36 performed on Homer called peaks. Following peak-calling, putative enhancers were
37 identified in the following way: regions of up to 3 kb flanked by H3K27ac and devoid

of H3K27me3 peaks were identified and assigned to the nearest gene using gene annotations from Ensembl (Galgal4.71) and refGene (Nov. 2011 ICGSC Gallus_gallus-4.0/galGal4). Read distributions around transcription start site (TSS) or the centre of a putative enhancer was plotted using 'annotatePeaks'. Putative enhancers for +FGF2-treated and control samples were compared to find common and unique putative enhancers using the R package ChIPpeakAnno (Zhu et al., 2010). Putative enhancers in +FGF2 and -FGF2 were considered to be overlapping and common if they had a 0 bp gap between them, otherwise they were considered to be unique to the respective condition. Genes associated with unique +FGF2 and -FGF2 putative enhancers were subjected to Gene Ontology (GO) analysis using DAVID (DAVID Bioinformatics Resources 6.7) (Huang et al., 2009a, b). All ChIP-seq data were viewed in the IGB browser (Nicol et al., 2009).

Transcription factor binding site analysis

Transcription factor binding site analysis was carried out using RSAT peak-motifs (Thomas-Chollier et al., 2012a; Thomas-Chollier et al., 2012b) and JASPAR (Sandelin et al., 2004) and TRANSFAC (Matys et al., 2006) libraries. To obtain significant binding sites, an e-value ≤ 0.05 was considered.

Conservation

Multiple alignments between 21 amniotes were obtained from Ensemble PECAN (Paten et al., 2008). Additionally, DREiVe (Khan et al., 2012), a motif-discovery algorithm was used to identify putative regulatory regions 300 KB upstream and downstream of selected FGF-response genes. It reports regulatory regions as clusters of short conserved motifs of 8 bp in a 300 bp window. DREiVe does not depend on sequence alignment, is able to identify re-arrangements of motifs within regulatory elements and does not require prior information of transcription factor binding sites. Regions that were conserved in 7 out of 9 species (human, horse, cow, rabbit, mouse, opossum, platypus, chick and lizard) were considered as putative enhancers. These tracks were then loaded into IGB browser to view with Chip-seq data.

FIGURE LEGENDS

Figure 1. Distinct regulatory element usage during OEP induction by FGF2.

(A) posterior sensory progenitor explants were dissected from 0ss (HH6) embryos and cultured in the presence (+FGF2) or absence (Cnt) of FGF2 for 6 hrs. Around 100 tissues per condition were processed for nanoChIP-seq. (B) Average read density of H3K27ac and H3K27me3 in control (pink) and +FGF2 treated cells (violet) around the transcription start site (TSS). Read densities are highest close to the TSS and display a peak-dip-peak pattern where the dip-region may represent bound transcriptional activators or repressors. (C) Bimodal distribution of H3K27ac (green) and absence/low H3K27me3 (grey) was used to identify enhancers. (D) In control (pink) and +FGF2 treated explants (violet), the average read density of H3K27ac and H3K27me3 around the centre of putative enhancers exhibit a bimodal distribution. H3K27me3 signals in both control and experimental cells is low, while H3K27ac is high as expected for active enhancers. (E) Bimodal H3K27ac peaks in control (Cnt; pink) and +FGF2 (violet) treated samples show a similar distribution with an average of around 170 located close to promoters, 130 in exonic, 1500 in intronic and 1700 in intergenic regions. (F) Venn-diagram representing putative enhancer elements unique in control (pink; 2,451) and +FGF2-treated sensory progenitors (violet; 2,883). 808 putative enhancers are shared in both conditions. (G) Putative enhancers were associated to the closest TSS. Gene ontology annotation reveals significant enrichment of ear and MAPK signalling related terms in enhancer associated genes in FGF2-treated sensory progenitors, but not in controls.

Figure2. Ear specific enhancers gain H3K27ac upon FGF stimulation of sensory progenitors.

IGB browser view of the Spry1 and Foxi3 locus (A, D), both expressed in OEPs, and of the FGF-repressed gene Pax6 (G) and the sensory progenitor gene Eya2 (H). H3K27ac tracks are shown in green and H3K27me3 tracks in grey. Enh: putative enhancers called by Homer and MACS2 (+FGF2 violet bars; control pink bars). Blue bars: Fos::Jun: location of AP1 binding motifs. CNS: conservation from PECAN alignments (ENSEMBL) and DREIVE are shown in red. (B, C) Spry1-E2 is active in OEPs and the otic cup. (E) Foxi3-E1 is active in OEPs at the 4-5ss (HH8), while Foxi3-E2 is activated later at the 10ss (HH10) in the non-otic ectoderm (NOE; F). (G) H3K27ac is lost in the Pax6 locus upon FGF treatment of sensory progenitor cells, while H3K27me3 is increased. The number of putative enhancers is reduced. (H) There are subtle changes in H3K27ac and H3K27me3 in the Eya2 locus. * marks an enhancer shared between controls and FGF2-treated cells.

Figure 3. AP1 binding sites are associated with gain of H3K27ac.

(A-B) Motif enrichment analysis using RSAT of enhancers active in control (Cnt; A) and FGF2 (B) treated sensory progenitors. Transcription factor families corresponding to the enriched motifs (M1-M30) are colour coded, $-\log(\text{evalue})$ is plotted on the y axis. (C) Expression levels of transcription factors corresponding to enriched motifs in the 3ss whole embryo (grey), in 0ss posterior sensory progenitors (green), 5-6ss OEPs (pink), 8-9ss OEPs (violet) and the 11-12ss otic placode (blue) from (Chen et al., 2017). (D) Jun::Fos (Ap1) Position Weight Matrix (PWM) logo; note: the Ap1 motif is highly enriched in FGF2-treated samples. (E) Distribution of H3K27ac peaks surrounding AP1 motifs in FGF2-treated and control sensory progenitors; SeqMINER view of the heatmaps. (F) H3K27ac peak profiles around the Ap1 motif in FGF2 treated (violet) and control (pink) sensory progenitors in a window of $\pm 5\text{kb}$.

Figure 4. Enhancer associated transcripts are regulated by FGF signalling.

(A) Sensory progenitor explants from HH6 chick embryos were cultured in the absence or presence of FGF2. After 6 hrs, the OEP marker *Pax2* is upregulated. (B) 3 hr FGF2 treatment promotes the expression of OEP transcripts, while repressing non-otic and late otic genes as determined by NanoString nCounter. A fold change of 1.5 or 0.25 (grey lines) and a p-value < 0.05 were used as threshold; transcripts not passing these thresholds are shown in grey and significantly up- and downregulated genes are shown in pink and violet, respectively. (C) After 6 hrs of FGF2 treatment sensory progenitors continue to express many 3hr-induced transcripts and upregulate new genes. A fold change of 1.5 or 0.25 (grey lines) and a p-value < 0.05 were used as threshold; transcripts not passing these thresholds are shown in grey and significantly up- and downregulated genes are shown in pink and violet, respectively. (D) Genes upregulated by FGF2 are expressed in OEPs, while FGF2-repressed genes are absent from OEPs (*Pax6*) as determined by in situ hybridisation.

Figure 5. Ap1 recruits p300 to OEP enhancers.

(A) De novo acetylation is required for OEP gene expression. Sensory progenitor explants from HH6 were cultured with FGF2 for 3 hrs in the presence of the p300 inhibitor C646 or vehicle control (DMSO). FGF2 fails to induce OEP genes when p300 activity is inhibited as assessed by qPCR. Red dots represent transcripts with

1 significant difference, grey dots no significant changes. (B) p300 occupies OEP
2 enhancers. Flag-tagged p300 together with a plasmid containing RFP (controls) or
3 dominant negative A-Fos (experimental) was electroporated into future OEPs at
4 HH6. After 6 hrs targeted otic placodes were dissected and ChIP was performed with
5 anti-Flag antibodies. p300 occupies *Spry1* and *Foxi3* enhancers active in OEPs, but
6 does not bind the enhancer for the muscle gene *MyoD*. In the presence of A-Fos
7 p300 binding is abolished on both OEP enhancers. (C) A model for OEP gene
8 activation in response to FGF signalling. See text for details.
9

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11 **COMPETING INTERESTS**

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13 The authors declare no competing or financial interests.

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15 **AUTHOR CONTRIBUTIONS**

16

17 A.S. designed the experiments together with M.T.; M.T. conducted most experiments
 18 and analysed the data together with A.S.; M.A. performed all bioinformatics analysis;
 19 M. Ahmed performed ChIP-qPCR in figure 5. M.T. and M. A. prepared the figures.
 20 A.S wrote the manuscript.

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A **B** **C** **D** **E** **F** **G****GO Cnt Enhancers** **GO +FGF2 Enhancers**

A **G** **D** **H**

Enriched motifs in Cnt enhancers

Enriched motifs in +FGF2 enhancers

C Cnt TFs +FGF2 TFs

D

E +FGF2 Cnt

F H3K27ac around Jun::Fos

A **B** **C** **D**

A **B** **C**