

1 **Calcium dynamics and chromatin remodelling underlie heterogeneity in**
2 **prolactin transcription**

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4 Short title: cellular heterogeneity in prolactin transcription

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6 Claire V Harper^{1*}, Anne V McNamara², David G Spiller², Jayne C. Charnock¹, Michael
7 RH White², Julian RE Davis³

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9 * Corresponding author

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11 ¹Department of Biology, Edge Hill University, Ormskirk, Lancashire, L39 4QP, UK.

12 ²Systems Microscopy Centre, and ³Endocrine Sciences Research Group, Faculty of

13 Biology, Medicine and Health, University of Manchester, Manchester, M13 9PT, UK

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15 *Corresponding author: harperc@edgehill.ac.uk

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24 Abstract

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26 Pituitary cells have been reported to show spontaneous calcium oscillations and dynamic
27 transcription cycles. To study both processes in the same living cell in real-time, we used
28 rat pituitary GH3 cells stably expressing human prolactin-luciferase or prolactin-EGFP
29 reporter gene constructs loaded with a fluorescent calcium indicator and measured
30 activity using single cell time-lapse microscopy. We observed heterogeneity between
31 clonal cells in the calcium activity and prolactin transcription in unstimulated conditions.
32 There was a significant correlation between cells displaying spontaneous calcium spikes
33 and cells showing spontaneous bursts in prolactin expression. Notably, cells showing no
34 basal calcium activity showed low prolactin expression but elicited a significantly greater
35 transcriptional response to BayK8644 compared to cells showing basal calcium activity.
36 This suggested the presence of two subsets of cells within the population at any one time.
37 Fluorescence-activated cell sorting was used to sort cells into two populations based on
38 the expression level of prolactin-EGFP however, the bimodal pattern of expression was
39 restored within 26h. Chromatin immunoprecipitation showed that these sorted
40 populations were distinct due to the extent of histone acetylation. We suggest that
41 maintenance of a heterogeneous bimodal population is a fundamental characteristic of
42 this cell type and that calcium activation and histone acetylation at least in part, drive
43 prolactin transcriptional competence.

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46

47 **Introduction**

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49 It is widely reported that transcription of genes is not a static process and can occur in
50 rapid bursts (with second - minute timescales (Blake, M, Cantor and Collins, 2003;
51 Fujita, Iwaki and Yanagida, 2016; Golding, Paulsson, Zawilski and Cox, 2005; Harper,
52 Finkenstadt, Woodcock, Friedrichsen, Semprini, Ashall, Spiller, Mullins, Rand, Davis et
53 al., 2011; Ozbudak, Thattai, Kurtser, Grossman and van Oudenaarden, 2002; Raj, Peskin,
54 Tranchina, Vargas and Tyagi, 2006; Raser and O'Shea, 2004; Yu, Xiao, Ren, Lao and
55 Xie, 2006) or longer cycles (with minute – hour timescales (Degenhardt, Rybakova,
56 Tomaszewska, Mone, Westerhoff, Bruggeman and Carlberg, 2009; Harper et al., 2011;
57 Molina, Suter, Cannavo, Zoller, Gotic and Naef, 2013; Suter, Molina, Gatfield,
58 Schneider, Schibler and Naef, 2011; Wijgerde, Grosveld and Fraser, 1995; Zenklusen,
59 Larson and Singer, 2008). In eukaryotic cells this has been studied at the population
60 biochemical level using chromatin immunoprecipitation to measure binding of
61 transcription factors to gene promoters (Kangaspeska, Stride, Metivier, Polycarpou-
62 Schwarz, Ibberson, Carmouche, Benes, Gannon and Reid, 2008; Metivier, Penot, Hubner,
63 Reid, Brand, Kos and Gannon, 2003), or in living single cells using reporter constructs or
64 direct RNA measurements visualise the kinetics of transcription (Chubb, Trcek, Shenoy
65 and Singer, 2006; Fritzsich, Baumgartner, Kuban, Steinshorn, Reid and Legewie, 2018;
66 Harper et al., 2011; Molina et al., 2013; Suter et al., 2011; White, Masuko, Amet, Elliott,
67 Braddock, Kingsman and Kingsman, 1995).

68

69 Transcription of the hormone prolactin (PRL) has been widely shown to be unstable and
70 pulsatile. The presence and timing of pulses is heterogeneous between cells in both
71 primary pituitary cells (Harper, Featherstone, Semprini, Friedrichsen, McNeilly, Paszek,
72 Spiller, McNeilly, Mullins, Davis et al., 2010; Semprini, Friedrichsen, Harper, McNeilly,
73 Adamson, Spiller, Kotelevtseva, Brooker, Brownstein, McNeilly et al., 2009; Shorte,
74 Leclerc, Vazquez-Martinez, Leaumont, Faught, Frawley and Boockfor, 2002) and clonal
75 pituitary cell lines (Castano, Kineman and Frawley, 1996; Harper et al., 2010; Harper et
76 al., 2011; Semprini et al., 2009; Takasuka, White, Wood, Robertson and Davis, 1998).
77 We have shown that activation of the human prolactin promoter occurs in long (~11h)
78 cycles and we calculated the duration of defined transcriptional ‘on’, ‘off’ and
79 ‘refractory’ periods within this cycle in transcriptionally active cells (Harper et al., 2011).
80 Histone acetylation was shown to be involved in generating these cycles. This supports
81 other studies that suggest that transcription bursts/cycles can be regulated by defined
82 periods of histone modification (Blake et al., 2003; Kangaspeska et al., 2008; Metivier et
83 al., 2003; Metivier, Reid and Gannon, 2006; Raj et al., 2006; Raser and O’Shea, 2004).
84
85 As well as the role of chromatin modifications on transcriptional heterogeneity, the link
86 between calcium signalling and transcription has been well reported. Studies from around
87 three decades ago showed that calcium was required for the transcription of prolactin
88 (Day and Maurer, 1990; Hoggard, Davis, Berwaer, Monget, Peers, Belayew and Martial,
89 1991; White, Bauerle and Bancroft, 1981). This was followed by pioneering work
90 showing that calcium dynamics are related to downstream transcription (Clapham, 2007;
91 Dolmetsch, Xu and Lewis, 1998). Primary pituitary cells and pituitary-derived cell lines

92 have been widely shown to exhibit spontaneous oscillations or spikes in intracellular
93 calcium concentration ($[Ca^{2+}]_i$) (Langouche, Roudbaraki, Pals and Deneff, 2001; Lewis,
94 Goodman, St John and Barker, 1988; Romano, McClafferty, Walker, Le Tissier and
95 Shipston, 2017; Schlegel, Winiger, Mollard, Vacher, Wuarin, Zahnd, Wollheim and
96 Dufy, 1987; Shorte, Faught and Frawley, 2000; Van Goor, Zivadinovic, Martinez-
97 Fuentes and Stojilkovic, 2001; Villalobos, Faught and Frawley, 1998; Wagner, Yacono,
98 Golan and Tashjian, 1993; Zimmer and Simasko, 2000) and a relationship has been
99 reported between the presence of calcium spikes and prolactin secretion (Charles, Piros,
100 Evans and Hales, 1999; Law, Pachter and Dannies, 1989; Van Goor et al., 2001). An
101 initial link between calcium spikes and prolactin transcription was suggested (Villalobos,
102 Nunez, Faught, Leaumont, Boockfor and Frawley, 2002) but is still not completely
103 understood.

104

105 In this study we focus on two factors that may contribute to the transcriptional
106 heterogeneity of prolactin seen within populations of pituitary cells; calcium dynamics
107 and histone modification.

108

109 **Materials and methods**

110

111 **Materials**

112 Fetal calf serum (FCS) was from Harlan Sera-Lab, Crawley Down, UK, Luciferin was
113 from Bio-Synth, Switzerland. BayK-8644phenyl methyl sulphonyl fluoride (PMSF) and

114 mammalian protease inhibitor cocktail were from Sigma, UK. Calcium indicator Fluo-4
115 and Calcium Orange-AM were from Invitrogen (USA).

116

117 **Production of stable cell lines and cell culture**

118 Clonal rat pituitary GH3 cells stably transfected with a 5kb hPRL-luciferase reporter
119 construct (GH3/prolactin-luc cells) or both the 5kb hPRL-luciferase and 5kb hPRL-
120 destabilised enhanced green fluorescent protein (d2EGFP) reporter constructs (GH3-
121 DP1) were used as described previously (Harper et al., 2011; Takasuka et al., 1998).
122 Cells were cultured in DMEM containing 10% v/v FCS and maintained at 37°C 5% CO₂.
123 Cells were maintained in antibiotic to avoid the loss of transgenes.

124

125 **Fluorescence and luminescence imaging**

126 GH3/prolactin-luc cells were seeded in 35-mm glass coverslip-based dishes (IWAKI,
127 Japan) 20h prior to imaging. Luciferin (1mM) was added at least 10h before the start of
128 the experiment, and the cells were transferred to the stage of a Zeiss Axiovert 200
129 equipped with an XL incubator (maintained at 37°C, 5% CO₂, in humid conditions)
130 maintained within a darkened room. Cells were loaded with Fluo-4 for 30 minutes and
131 then time-series imaging was performed using a Fluar x20, 0.75 NA (Zeiss) air objective,
132 with an Argon ion laser at 488nm. Emitted light was captured through a 505-550 nm
133 bandpass filter from a 545 nm dichroic mirror. Calcium recordings were captured every 1
134 second for at least 250 seconds unless stated otherwise. Data were captured using
135 LSM510 software with consecutive autofocus. The microscope and all light emitting
136 devices were then shut down and luminescence images were captured using a photon-

137 counting charge coupled device camera (Orca II ER, Hamamatsu Photonics, UK).
138 Sequential images, integrated over 30 min, were taken using 4 by 4 binning and acquired
139 using Kinetic Imaging software AQM6 (Andor, UK). Bright field images were taken
140 before and after luminescence imaging to allow localization of cells. In the relevant
141 experiments 0.5 μ M BayK8644 was added to the dish at around 100 sec during the
142 calcium imaging period.

143

144 **Analysis of imaging data**

145 Analysis was carried out using Kinetic Imaging AQM6 software (Andor, UK). Regions
146 of interest were drawn around each single cell, and mean intensity data were collected for
147 both the fluorescence and luminescence time-series. The average instrument dark count
148 (corrected for the number of pixels being used) was subtracted from the luminescence
149 signal.

150 Assessment for criteria of luminescence activity was determined as follows. In
151 unstimulated experiments, the luminescence values from each cell were normalised to the
152 population average. A cell that maintained normalised luminescence values lower than
153 the average (1 fold) was termed 'Low'. A cell that maintained normalised luminescence
154 values higher than the average (1 fold) or where the normalised luminescence values
155 varied across the average during the experiment was termed 'High'. In experiments
156 where the cells were stimulated with 0.5 μ M BayK8644, the luminescence values from
157 each cell were normalised to the average of the first two data points for that particular
158 cell. A response to stimulus (transcription rise) was recorded if the data points for that
159 particular cell increased within 3 hours and reached a 1.5 fold increase within 4 hours.

160 Data is presented as mean +/- SD and Mann-Whitney non-parametric tests are used, using
161 GraphPad Prism. Classification of active or inactive calcium was assessed manually,
162 where active calcium referred to cells showing calcium spikes within the 250s imaging
163 period. Traces were scored blind. Outlying data points were not excluded from the plots.

164

165 **Flow cytometry and fluorescence activated cell sorting (FACS)**

166 GH3-DP1 cells were trypsinised and re-suspended in phosphate-buffered saline (PBS) at
167 a concentration of 10^6 cells/ml, before analysis by flow cytometry using a Coulter-Epics
168 Altra flow cytometer. 10,000 cells/sample were analysed. Cells were sorted for low and
169 high expression of prolactin-d2EGFP using FACS with wildtype GH3 cells used to detect
170 autofluorescence levels. A sample of sorted low and high cells were plated into non-
171 adherent dishes and analysed again after 26h. For ChIP experiments, at least 1.5×10^6 cells
172 were collected for each of the low, high, unsorted and IgG (unsorted) samples.

173

174 **ChIP assays and RT-PCR**

175 Experiments using FACS sorted GH3-DP1 cells (1.5×10^6 per sample) were carried out
176 immediately with the cells in suspension. Formaldehyde was added to each tube at a final
177 concentration of 1% v/v and incubated at room temperature for 15 min. Tubes were kept
178 on ice and then samples were washed twice by centrifugation with PBS supplemented
179 with protease inhibitors (1 mM PMSF and 1x mammalian protease inhibitor cocktail).
180 Cells were resuspended into 500 μ l of PBS with protease inhibitors, centrifuged (4 min,
181 2000 rpm at 4°C) and the pellet resuspended in 200 μ l SDS lysis buffer as described
182 previously (Ashall, Horton, Nelson, Paszek, Harper, Sillitoe, Ryan, Spiller, Unitt,

183 Broomhead et al., 2009) based on the protocol by Upstate Biotechnology.
184 Immunoprecipitation was carried out using 5 µg of either anti-acetylated H3 (Merck
185 Millipore #06-599), anti-IgG (Merck Millipore #12-370) or anti-Pit-1 (Santa Cruz #X-7)
186 antibody. DNA was extracted and amplified by PCR as described previously (Ashall et
187 al., 2009). The primer sequences used were: prolactin Promoter1 left
188 GCAATCTTGAGGAAGAACTTGA, right AGGCATTCGTTTCCCTTTTC
189 amplifying 347bp of DNA; prolactin Promoter2 left GCATGGGAACTTTAGCATCA,
190 right ATAGCCCCACATTTCTGTG amplifying 351bp; prolactin Promoter3 left
191 CCTGTGCACATGGACAGAAT, right CCATAGTGGAAAGCATTTGGAA amplifying
192 358bp. PCR products were resolved using agarose gel electrophoresis and densitometry
193 was performed using AQM Advance 6.0 software (Kinetic Imaging, UK). Values were
194 normalised to the unstimulated sample.

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204 **Results**

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206 **Temporal variation in basal prolactin transcription and calcium patterns in GH3**
207 **cells**

208 Pulses in prolactin transcription have been reported for many years (Featherstone, Hey,
209 Momiji, McNamara, Patist, Woodburn, Spiller, Christian, McNeilly, Mullins et al., 2016;
210 Harper et al., 2010; Harper et al., 2011; Semprini et al., 2009; Shorte et al., 2002;
211 Takasuka et al., 1998). In previous work using luminescent and fluorescent microscopy
212 of reporter gene constructs we described the evidence of clearly defined prolactin
213 transcription cycles in single cells, occurring approximately every 11-12h (Harper et al.,
214 2011). These cycles are observed in clonal GH3 cells and also in transgenic primary
215 pituitary cells (with a longer cycle of ~15h) using reporter constructs of varying promoter
216 length (Harper et al., 2011).

217

218 Detailed analysis of prolactin transcriptional activity in GH3 cells containing a 5kb
219 prolactin promoter-luciferase reporter gene (GH3/prolactin-luc cells) showed that 2
220 transcriptional patterns occurred in unstimulated (basal) conditions: ~35% of cells
221 maintained a relatively even low level of luminescence signal, whereas ~65% showed
222 high or cycling signal over a recorded 10 hour period of imaging (Figure 1A,B; 91 cells,
223 6 experiments). This analysis is in agreement with our previous study where ~50% of
224 cells were recorded to show transcription cycles as detected using a binary model of
225 transcription switch times (Harper et al., 2011).

226

227 GH3 cells, along with other pituitary derived cells, have been widely shown to exhibit
228 spontaneous calcium oscillations (Lewis et al., 1988; Schlegel et al., 1987; Shorte et al.,

229 2000; Villalobos et al., 1998; Wagner et al., 1993; Zimber and Simasko, 2000). Using
230 GH3/prolactin-luc cells loaded with the calcium indicator Fluo-4 to measure changes in
231 intracellular calcium ($[Ca^{2+}]_i$), we detected spontaneous calcium spikes in around 60% of
232 cells within a 250 sec period of imaging (Fig. 1C,D). Patterns varied between cells in the
233 timing of the spikes (Fig. 1D). Approximately 30% of cells showed no calcium spikes
234 within the period of imaging although these cells maintained low basal level of
235 fluorescence above background levels.

236

237 **Relationship between calcium dynamics and prolactin transcription profiles in** 238 **single cells**

239 A key question that arose from these observations was whether there is a relationship
240 between the basal calcium signal and the basal prolactin expression within a particular
241 cell. To answer this, fields of adherent GH3/prolactin-luc cells were loaded with Fluo-4
242 to measure $[Ca^{2+}]_i$ and fluorescent images were captured every 1 sec for up to 300 sec.
243 Then subsequently, luminescence images were captured on the same field of cells to
244 record prolactin promoter activation (Fig. 2A). $[Ca^{2+}]_i$ profiles were divided into inactive
245 (those showing no calcium spikes within the period of imaging) or active (those showing
246 any form of calcium oscillations) (Fig.2B). Luminescence profiles were divided into 2
247 categories; low and high as described in figure 1. It was clearly apparent that there was a
248 relationship between the $[Ca^{2+}]_i$ profile and the transcriptional state of the cells (Fig.
249 2B,C). ~80% of cells showing no calcium spikes showed low maintained levels of
250 prolactin transcription for at least 10h after the calcium recordings were generated. In
251 cells showing active calcium oscillations, over 80% were displaying high prolactin

252 expression during the following 10 hours. This difference was highly significant (Fig. 2C;
253 $p < 0.001$ t-test; 6 experiments, 91 cells). These data suggest that $[Ca^{2+}]_i$ may prime a cell
254 for transcriptional activation, or that the $[Ca^{2+}]_i$ profile determines transcriptional
255 competence of a particular cell.

256

257 **Relationship between calcium dynamics and the prolactin transcriptional response** 258 **to stimulus**

259 Previous research has shown that the transcription of prolactin is cyclical in basal
260 conditions (Harper et al., 2011). These cycles are composed of an active ‘on’ phase of
261 transcriptional activation (approximately 4h), and an inactive ‘off’ phase of
262 transcriptional inactivation (approximately 6.5h). The ‘off’ phase also contains a
263 refractory period of chromatin modelling ($>3h$) where cells cannot respond to stimulus
264 (Harper et al., 2011). Therefore the hypothesis is that cells can only respond immediately
265 to stimulus within the non-refractory period of the ‘off’ phase. Application of the acute
266 inducer of $[Ca^{2+}]_i$ increase, BayK8644, caused a rise in prolactin transcription in $43 \pm 3\%$
267 of cells ($n=5$ experiments, 77 cells) within the first 3 hours following treatment. This
268 supports the above hypothesis in that not all cells are in a state in which they can be
269 activated immediately. To test whether the transcriptional response to stimulus varied
270 depending on the preceding basal $[Ca^{2+}]_i$ profile of the cell, GH3/prolactin-luc cells were
271 labelled with fluo-4 and imaged for changes in $[Ca^{2+}]_i$, during which $0.5 \mu M$ BayK8466
272 was applied to the dish. Prolactin transcription was then measured in the same field of
273 cells for up to 10h.

274

275 Although BayK8644 induced an increase in $[Ca^{2+}]_i$ in most cells, there was a surprising
276 relationship between the basal $[Ca^{2+}]_i$ profile of a cell before stimulus and its
277 transcriptional response to the stimulus (Fig. 3). The majority of cells where no basal
278 oscillations in $[Ca^{2+}]_i$ were recorded prior to stimulus addition responded with a
279 significant transcriptional rise following application of the stimulus (Fig. 3A,C,D; for
280 determination of a significant transcriptional rise see Methods). In cells showing basal
281 oscillations in $[Ca^{2+}]_i$ before addition of the stimulus, few responded with a stimulus-
282 induced transcriptional rise (Fig. 3B,C,D). This difference was highly significant (Fig.
283 3D; $67\pm 10\%$ in inactive cells compared to $26\pm 8\%$ in active cells; $p < 0.01$, t-test, 5
284 experiments, 77 cells).

285

286 These data, taken together with those of Fig. 2, suggest that cells showing basal
287 oscillations in $[Ca^{2+}]_i$ are the prolactin-transcriptionally active population but are less able
288 to respond immediately to acute application of stimulus. In contrast, cells showing no
289 basal $[Ca^{2+}]_i$ oscillations are transcriptionally dormant (within our experimental detection
290 range) but poised to generate an immediate transcriptional response to the calcium
291 stimulus.

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295 **Temporal heterogeneity in prolactin transcription in clonal GH3 cells**

296 Several reports using reporter gene constructs have shown that clonal and primary
297 pituitary cells display heterogeneity in the levels of human prolactin expression (Castano

298 et al., 1996; Featherstone, Harper, McNamara, Semprini, Spiller, McNeilly, McNeilly,
299 Mullins, White and Davis, 2011; Harper et al., 2010; Harper et al., 2011; Semprini et al.,
300 2009; Shorte et al., 2002; Takasuka et al., 1998). To estimate the extent of basal cellular
301 heterogeneity, GH3 cells stably expressing prolactin-d2EGFP were analysed using flow
302 cytometry (Fig. 4A). Wildtype GH3 cells were used as an auto-fluorescence control. The
303 reporter gene fluorescence intensity within the unstimulated cell population varied over
304 two orders of magnitude indicating cellular variation in the expression of prolactin (Fig.
305 4B). The distribution of the cell population was bimodal suggesting that there may be 2
306 dominant groups of cells, high prolactin expression and low prolactin expression. We
307 have previously shown that cells switch from a transcription ‘on’ state to an ‘off’ state in
308 unstimulated conditions over the duration of several hours (Harper et al., 2011) so these
309 data support that view. Using fluorescence-activated cell sorting, the cells were sorted
310 into two populations; ‘Low’ (~30% of the total population) and ‘High’ (~70% of the total
311 population). The fluorescence levels of these sorted populations were re-analysed after 1h
312 and 26h to measure the dynamic responsiveness of individual cells (Fig. 4A,C-E). After
313 26h, the High cell population maintained a similar distribution. But in contrast, the Low
314 population of cells had changed, reverting back into the bimodal distribution shown in the
315 unsorted population (Fig. 4D,E). This clearly shows that the fluorescence expression
316 level of the cells is transient, with cells capable of switching between low and high
317 transcriptional states.

318

319 **Relationship between prolactin transcription and histone modification status**

320 We have previously suggested that the cycles in prolactin transcription are modulated by
321 histone acetylation, in particular proposing that the refractory period of transcriptional
322 activation may be the result of a period of closed chromatin (Harper et al., 2011). To
323 determine in more detail whether the extent of histone acetylation changes during
324 prolactin transcription cycles, GH3-DP1 cells were sorted into populations of Low and
325 High basal prolactin expression by FACS as described above (Fig. 4A). Chromatin
326 immunoprecipitation (ChIP) was immediately performed on these cell populations, using
327 unsorted GH3-DP1 cells as a comparison. Three sites were selected within the human
328 prolactin promoter to measure the extent of acetylated histone H3 (Ac-H3) bound to the
329 DNA (Fig. 5A). The localisation of these sites was based on the prior knowledge that
330 there are enhancer regions within this promoter (Peers, Voz, Monget, Mathy-Hartert,
331 Berwaer, Belayew and Martial, 1990; Van De Weerd, Peers, Belayew, Martial and
332 Muller, 2000). Primer 1 was in the proximal enhancer region, primer 2 was 2kb upstream
333 and primer 3 was in the distal enhancer region 4kb upstream. All three regions contained
334 Pit-1 binding sites, the critical transcription factor for prolactin expression (Fig. 5A). In
335 the Low prolactin transcription cell population (also containing cells in a transcriptional
336 refractory phase (Harper et al., 2011)) there was a decrease in Ac-H3 bound to all three
337 sites in the human prolactin promoter when compared to transcriptionally High
338 population of cells (Fig. 5B,C). This implies that the chromatin was more accessible
339 during periods of high prolactin transcription. In contrast, the extent of Pit-1 binding
340 remained consistent across the low and high prolactin transcriptional cell populations.
341 (Supplementary Figure 1), suggesting that Pit-1 remains bound to the DNA during cycles

342 of prolactin transcription in unstimulated conditions and that the cycles in transcription
343 are not due to cycles in Pit-1 binding.

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365 **Discussion**

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367 Cycles in prolactin gene expression have been well reported in the literature
368 (Featherstone et al., 2016; Harper et al., 2010; Harper et al., 2011; Semprini et al., 2009;
369 Shorte et al., 2002) but here we add new mechanistic information about how these cycles
370 may occur. We show that within a clonal population of resting GH3 cells there is
371 variability in the extent of prolactin expression, calcium dynamics and histone
372 acetylation. The resting calcium dynamics appear to determine the transcriptional
373 competence of the cell, i.e. whether a cell is transcriptionally active or can respond to a
374 stimulus. Within the population of GH3 cells there were two distinct subpopulations; 1)
375 cells showing inactive calcium, low prolactin transcription and decreased Ac-H3 binding
376 on the human prolactin promoter (closed chromatin) and 2) cells showing active calcium,
377 high or cycling prolactin transcription and increased Ac-H3 binding on the prolactin
378 promoter (open chromatin) (Fig. 6A). In contrast, the levels of Pit-1 binding to the human
379 prolactin promoter were not related to the degree of prolactin transcription implying that
380 Pit-1 may remain bound to the DNA and be controlled by post-translational modifications
381 (Demarco, Voss, Booker and Day, 2006).

382

383 Work from other groups has suggested a role for calcium signalling in the epigenetic
384 regulation of genes. Sharma and colleagues described a mechanism where increased
385 calcium levels led to changes in chromatin modifications and regulation of gene
386 expression at the level of alternative splicing in cardiomyocytes (Sharma, Nguyen, Geng,
387 Hinman, Luo and Lou, 2014) and Raynal et al. interestingly showed the potential

388 importance of calcium signalling on the reversal of epigenetic silencing of tumour
389 suppressor genes (Raynal, Lee, Wang, Beaudry, Madireddi, Garriga, Malouf, Dumont,
390 Dettman, Gharibyan et al., 2016). In light of this work, further study should be carried out
391 to determine whether the levels of $[Ca^{2+}]_i$ set up a cell for transcriptional activation by
392 mechanisms involving chromatin remodelling. Following our earlier work, where we
393 showed that the histone deacetylase inhibitor Trichostatin A affected basal prolactin
394 expression dynamics (Harper et al., 2011), it would be interesting to determine whether
395 the relationship between calcium and transcriptional activity can be modulated by
396 disrupting chromatin remodelling.

397

398 Maintenance of cellular heterogeneity has been reported to be functionally advantageous
399 at the population level (Paszek, Ryan, Ashall, Sillitoe, Harper, Spiller, Rand and White,
400 2010). We hypothesise that maintenance of a heterogeneous cell population is of innate
401 importance in these hormone producing cells and that the variability in transcription
402 correlated with variability in the calcium status and histone modification status of the
403 cells. Heterogeneity within the cell population was disrupted by separating into two cell
404 populations based on the level of prolactin gene expression. The observation that the low
405 cell population reverted back to having the same transcriptional distribution as the
406 unsorted population within 26h implies that cells are not constrained to one pattern of
407 expression (high or low), and can switch between states, potentially dependent on the
408 surrounding cells. This observation of maintenance to a steady-state population
409 distribution supports other reports in other clonal cell lines (Pilbrough, Munro and Gray,

410 2009; Sigal, Milo, Cohen, Geva-Zatorsky, Klein, Liron, Rosenfeld, Danon, Perzov and
411 Alon, 2006) although this appears to occur more rapidly in our cells.

412

413 The maintenance of a heterogeneous cell population may be important within pituitary
414 tissue, whereby at any fixed time there is a subset of cells expressing prolactin to enable
415 low, chronic basal hormone production (transcriptionally high and cycling cells) but there
416 is also a subset of cells which are ready to mount a response to external stimuli to enable
417 acute hormone production (transcriptionally low cells; Fig. 6B). The observation that an
418 external stimulus (BayK8644) induced prolactin transcription in significantly more
419 calcium inactive cells compared to calcium active cells provided further evidence that
420 there are two cellular sub-populations and supports the idea that it is the inactive cells
421 that are capable of mounting a rapid rise in prolactin transcription. Using similar
422 simultaneous measurements of calcium and rat PRL-luciferase expression in primary rat
423 mammotropes, Villalobos and colleagues (Villalobos et al., 2002) showed that the extent
424 of transcriptional response to TSH-releasing hormone was dependent on the resting
425 transcriptional status and the profile of $[Ca^{2+}]_i$ response. Whether our observations occur
426 in primary rat pituitary cells has not been determined in this study. Heterogeneity in
427 $[Ca^{2+}]_i$ has also recently been reported in corticotroph cell populations following
428 treatment with the hypothalamic secretagogues corticotrophin-releasing hormone and
429 arginine vasopressin (Romano et al., 2017). Our results, together with the findings from
430 these other studies, suggest that cell variability may be mechanistically important at the
431 population level within endocrine tissues, enabling graded responses to varying
432 stimulation levels through changes in cell recruitment. Whether there is a relationship

433 between $[Ca^{2+}]_i$, prolactin transcription and secretion, namely whether cells with inactive
434 calcium and low transcription are non-secreting, remains to be shown.

435

436 In summary we report, for the first time, a significant relationship between the basal
437 calcium dynamics and prolactin transcription in single living GH3 rat pituitary cells. We
438 also show that variability in the extent of histone acetylation on the prolactin promoter
439 determines basal prolactin transcription. It remains to be studied how the heterogeneity
440 within the pituitary cell population is maintained and whether these cells are capable of
441 detecting the status of surrounding cells (through paracrine signalling) and adjusting their
442 role accordingly.

443

444 **Declaration of Interest**

445 The authors have no declaration of interest.

446

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451

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454 the manuscript. Hamamatsu Photonics and Carl Zeiss Limited provided technical support.

455

456 **Author Contribution Statement**

457 CVH designed, performed and analysed the research and wrote the manuscript. AVM led
458 the FACS experiments and advised on experiments throughout the study. JC advised on
459 the manuscript. DGS was involved in discussions on the analysis and the manuscript, and
460 managed the Systems Microscopy Centre. MRHW directed the Systems Microscopy
461 Centre and was involved in critical discussions of the work. JRED advised throughout the
462 study and assisted with writing the manuscript.

463

464 **Figure Legends**

465

466 **Figure 1 – Temporal heterogeneity in prolactin transcription and calcium profiles**

467 **between pituitary cells.** (A,B) GH3 cells stably expressing a 5kb prolactin-luciferase
468 reporter gene (GH3/prolactin-luc cells) show 2 transcription patterns in unstimulated
469 conditions; low and high (see methods for classification), measured using time-lapse
470 luminescence imaging. Each line represents a single cell, thick black line is experiment
471 average. (C,D) GH3/prolactin-luc cells loaded with Fluo-4 show both inactive and active
472 calcium patterns in unstimulated conditions measured using time-lapse fluorescence
473 imaging. Each line represents a single cell. Scatter plots show the proportion of cells
474 defined by each category in unstimulated conditions where each point represents a single
475 experiment (B,D right panels). Bars in image series represent 50µm.

476

477 **Figure 2 – Relationship between calcium patterns and prolactin transcription in**

478 **pituitary cells in unstimulated conditions.** (A,B) Resting calcium profiles and prolactin

479 transcription were measured sequentially in the same cells. (B) Representative cells
480 showing inactive and active calcium patterns and their subsequent transcriptional
481 patterns. Right panels show mean prolactin transcriptional activity from all cells within
482 an experiment that show inactive or active calcium +/- SD. (C) Scatter plot shows the
483 proportion of cells exhibiting low or high prolactin transcription following active or
484 inactive calcium profiles (6 experiments, 91 cells; $p < 0.01$) where each point represents a
485 single experiment. Bar in image represents $20\mu\text{m}$.

486

487 **Figure 3 - Relationship between calcium patterns and prolactin transcription in**
488 **pituitary cells in stimulated conditions.** (A,B) Calcium profiles and subsequent
489 prolactin transcriptional response patterns following treatment with $0.5\mu\text{M}$ BayK8644.
490 The calcium and transcriptional responses to $0.5\mu\text{M}$ BayK8644 were measured in cells
491 that showed initial (pre-stimulus) active (A) or inactive (B) resting calcium profiles. Red
492 gene expression traces show a response and black traces show no response to the stimulus
493 (see methods for classification). (C) Mean single cell transcriptional response patterns
494 from cells showing initial active or inactive calcium profiles. Points show mean +/- SD.
495 (D) The proportion of cells showing transcriptional response to stimulus following initial
496 active or inactive calcium profiles, mean +/-SD (5 experiments, 77 cells, $p < 0.01$) where
497 each point represents a single experiment.

498

499 **Figure 4 – Maintenance of heterogeneity between clonal cells.** (A) Model showing
500 protocol. GH3 cells stably expressing a 5kb prolactin-destabilised EGFP reporter gene
501 (GH3-DP1 cells) were sorted for basal prolactin expression level using FACS. The

502 fluorescence of these sorted cell populations was then measured after 1h and 26h. (B)
503 Variation in basal prolactin gene expression in clonal GH3-DP1 cells (green trace)
504 compared to the wildtype GH3 cell line (black trace). Measurement of fluorescence
505 levels in High (blue trace) and Low (red trace) expressing GH3-DP1 cells following
506 FACS after 1h (C) and 26h (D). Data from one representative experiment are shown. (E)
507 Table showing the proportion of cells +/-SD classified as High or Low prolactin
508 expression 1h and 26h post-FACS in GH3 cells (control), unsorted cells, low expressing
509 cell population and high expressing cell population (3 experiments).

510

511 **Figure 5 – Relationship between level of prolactin transcription and chromatin**
512 **status at the prolactin promoter.** (A) Location of target sites for amplification within
513 the proximal prolactin promoter (designated P1, P2 and P3). GH3-DP1 cells expressing
514 prolactin-eGFP were sorted by level of basal prolactin transcription using FACs (see
515 Figure 4). Cells were classified as unsorted (Un), low transcription (Low) and high
516 transcription (High). (B,C) The level of Acetylated histone H3 was measured using ChIP
517 across the three amplification sites (2 experiments, mean +/-SD).

518

519 **Figure 6 – Schematic showing cellular heterogeneity in single pituitary cells and**
520 **pituitary cells within a tissue.** (A) Relationship between calcium profile, prolactin
521 transcription and chromatin status in single pituitary cells. (B, top panel) In basal
522 conditions a subset of cells within pituitary tissue is expressing prolactin at any one time,
523 resulting in low, chronic basal expression of prolactin across the tissue. (B, bottom panel)

524 In stimulated conditions, the cells showing low prolactin transcription within the tissue
525 respond to the stimulus, mounting an acute surge of prolactin expression.

526

527 **Supplementary Figure 1** - GH3-DP1 cells expressing prolactin-EGFP were sorted by
528 level of basal prolactin transcription using FACs (see Figure 4). Cells were classified as
529 unsorted (Un), low transcription (Low) and high transcription (High). (B,C) The level of
530 Pit-1 was measured using ChIP across the three amplification sites described in Figure 5.

531

532

533

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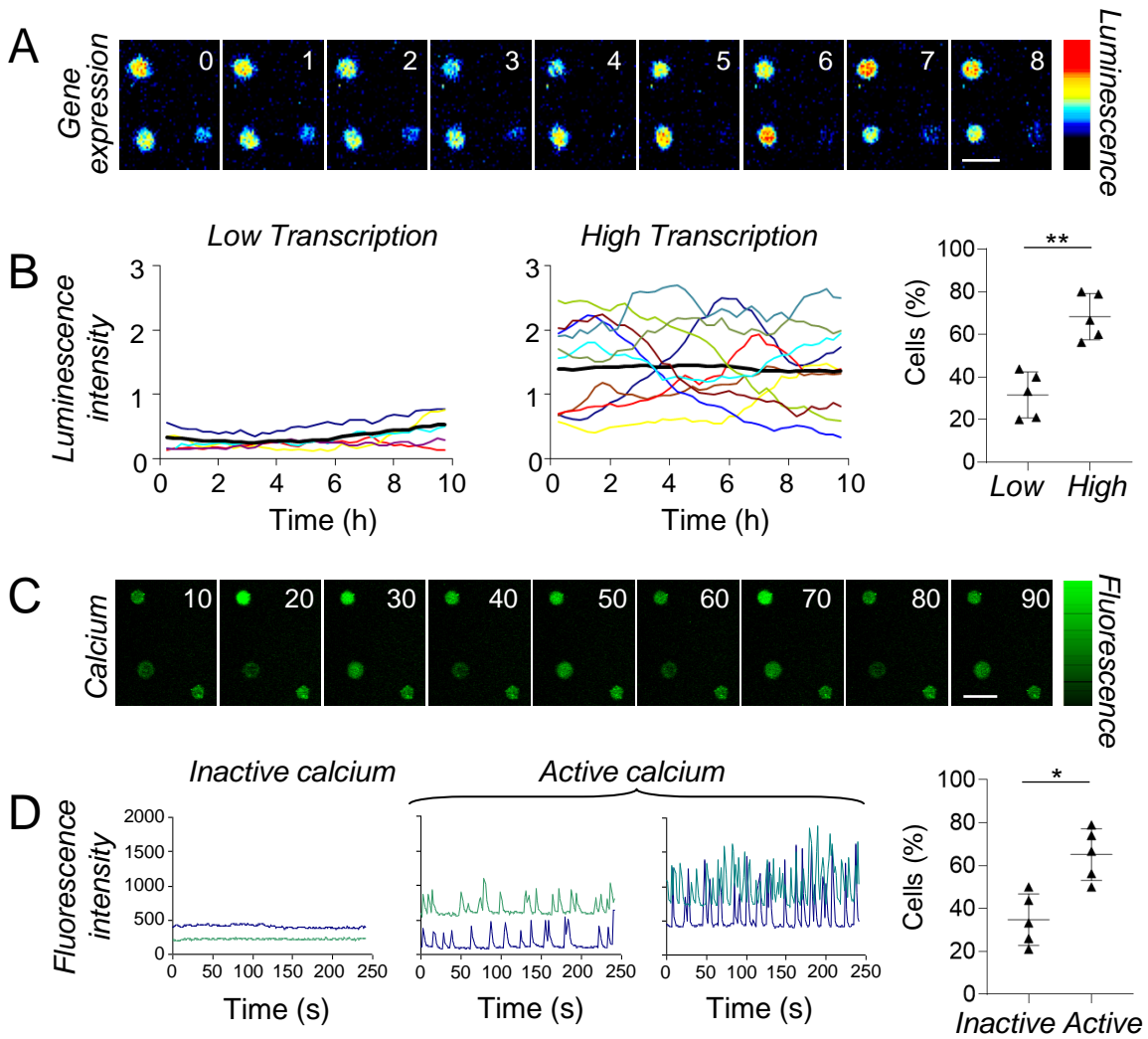


Figure 1

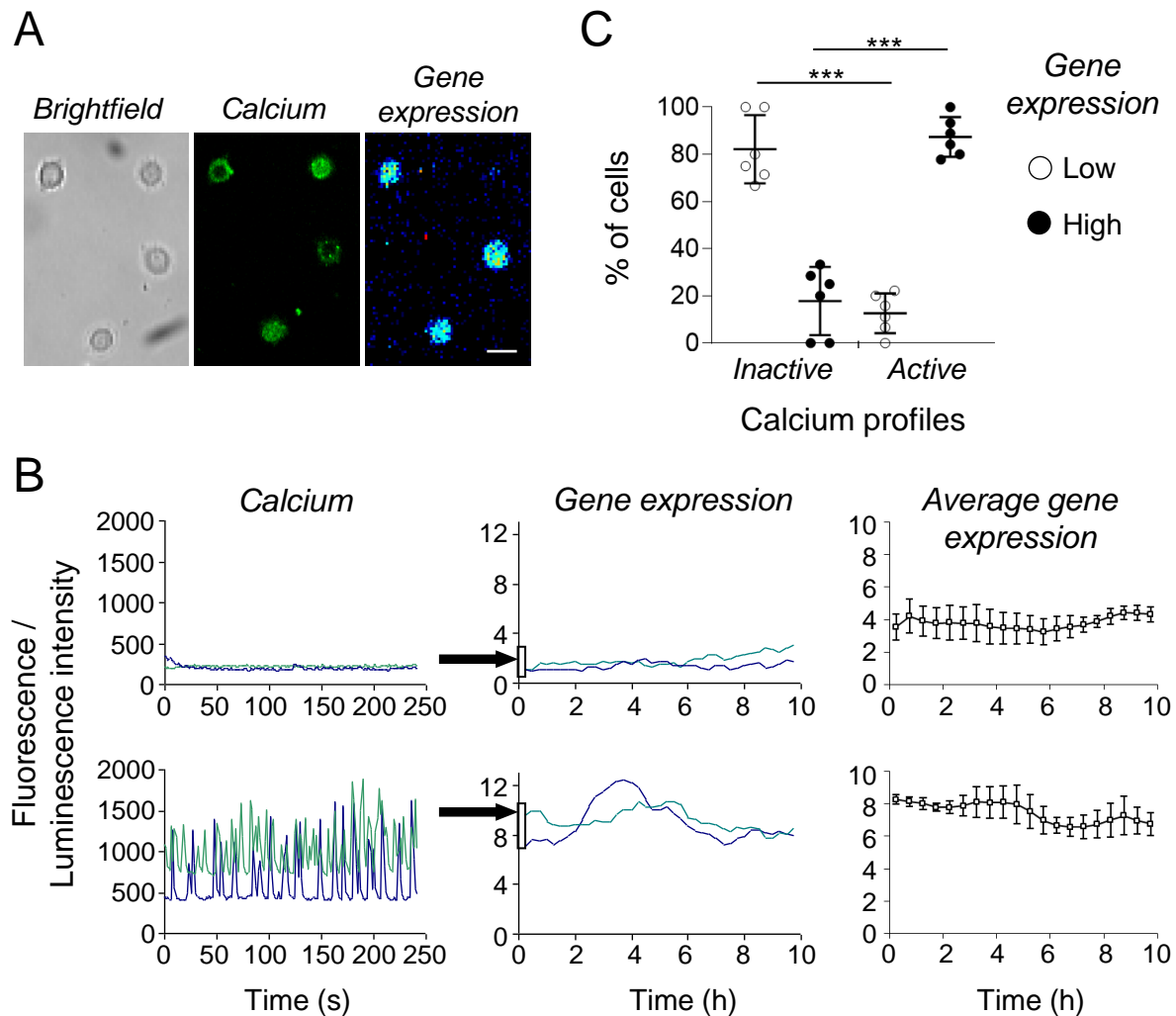


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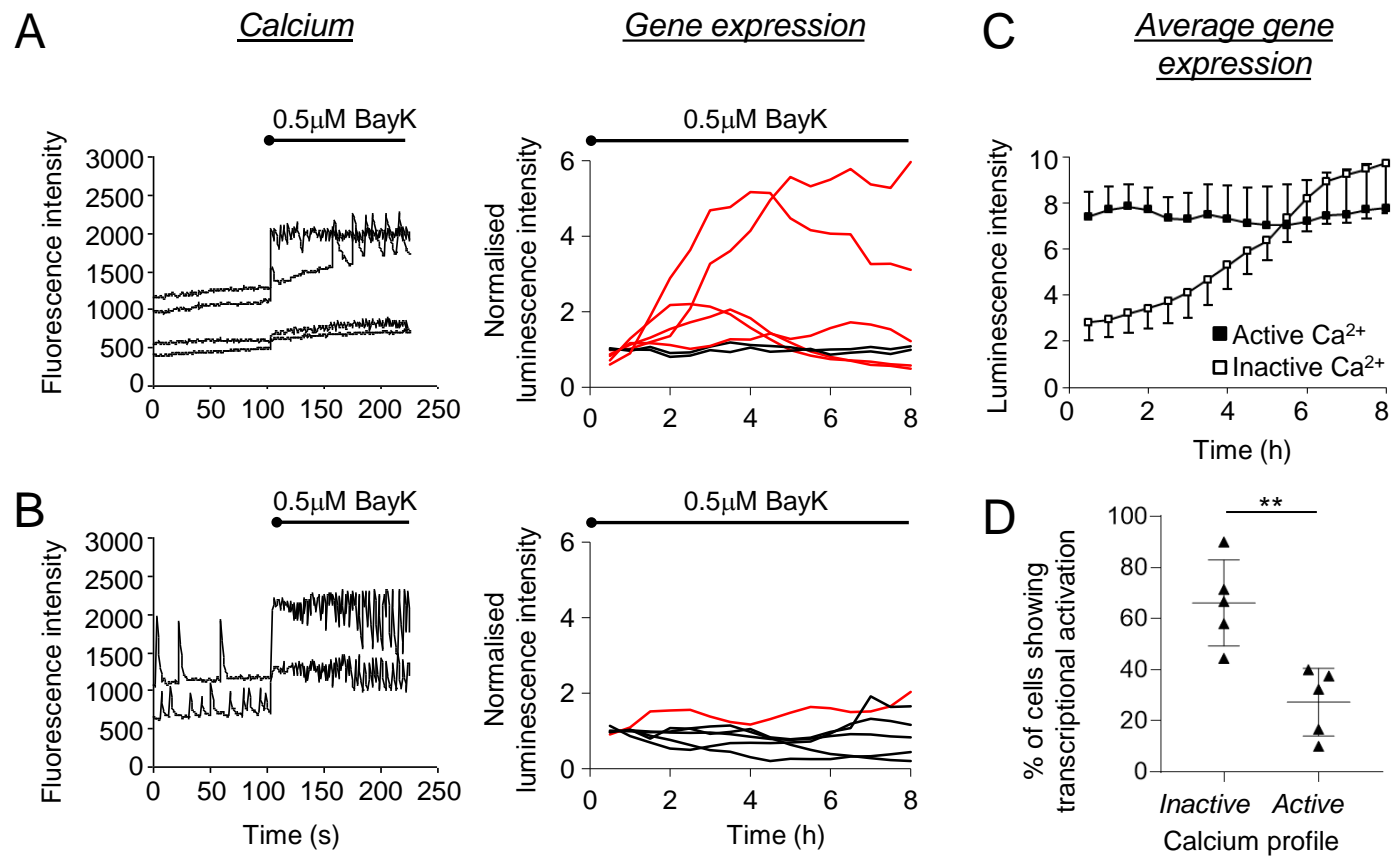


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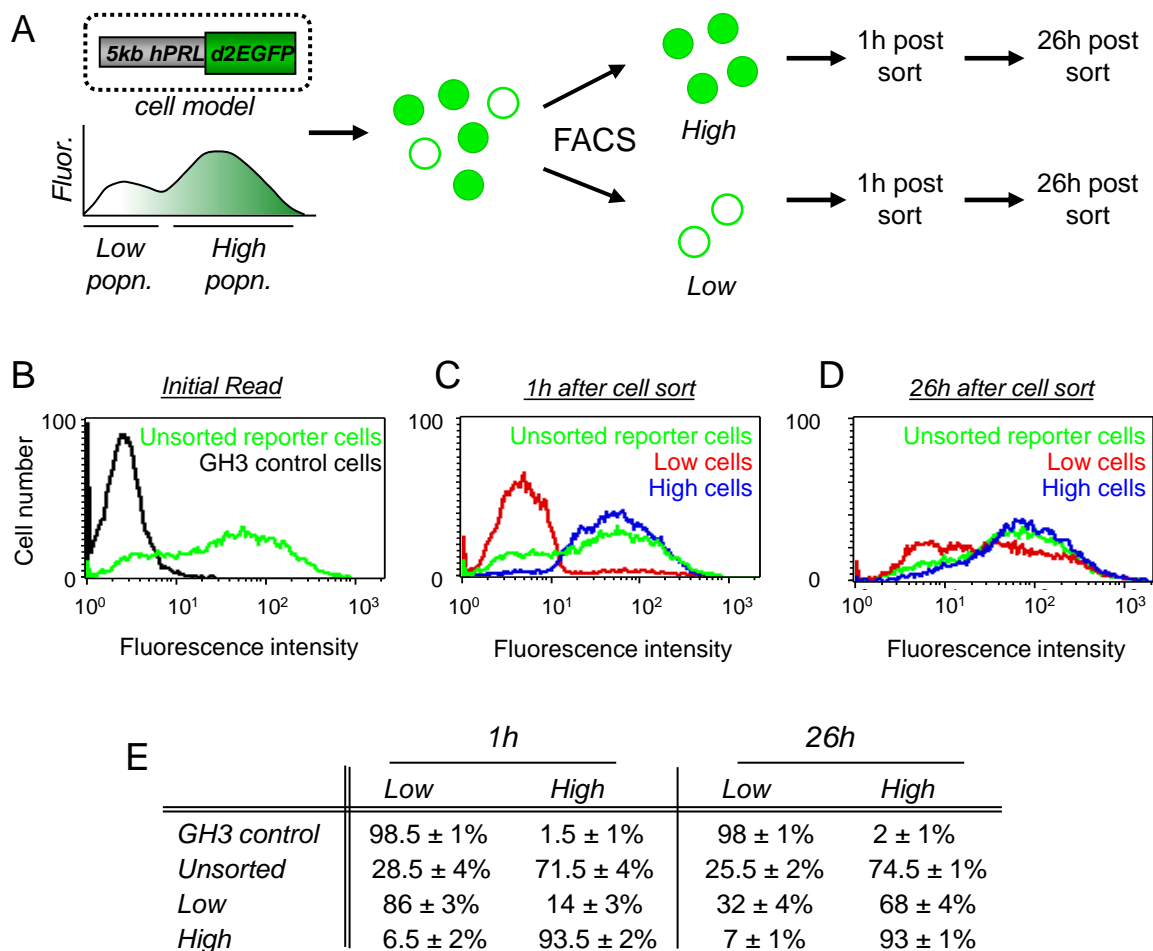


Figure 4

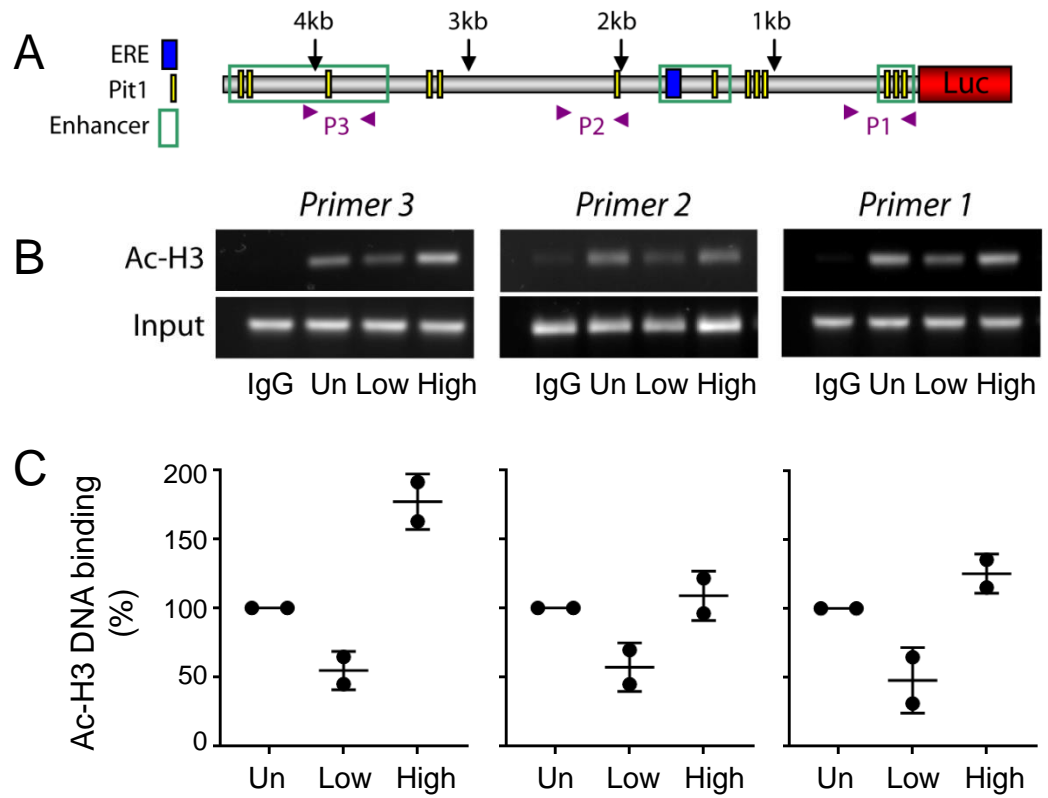


Figure 5

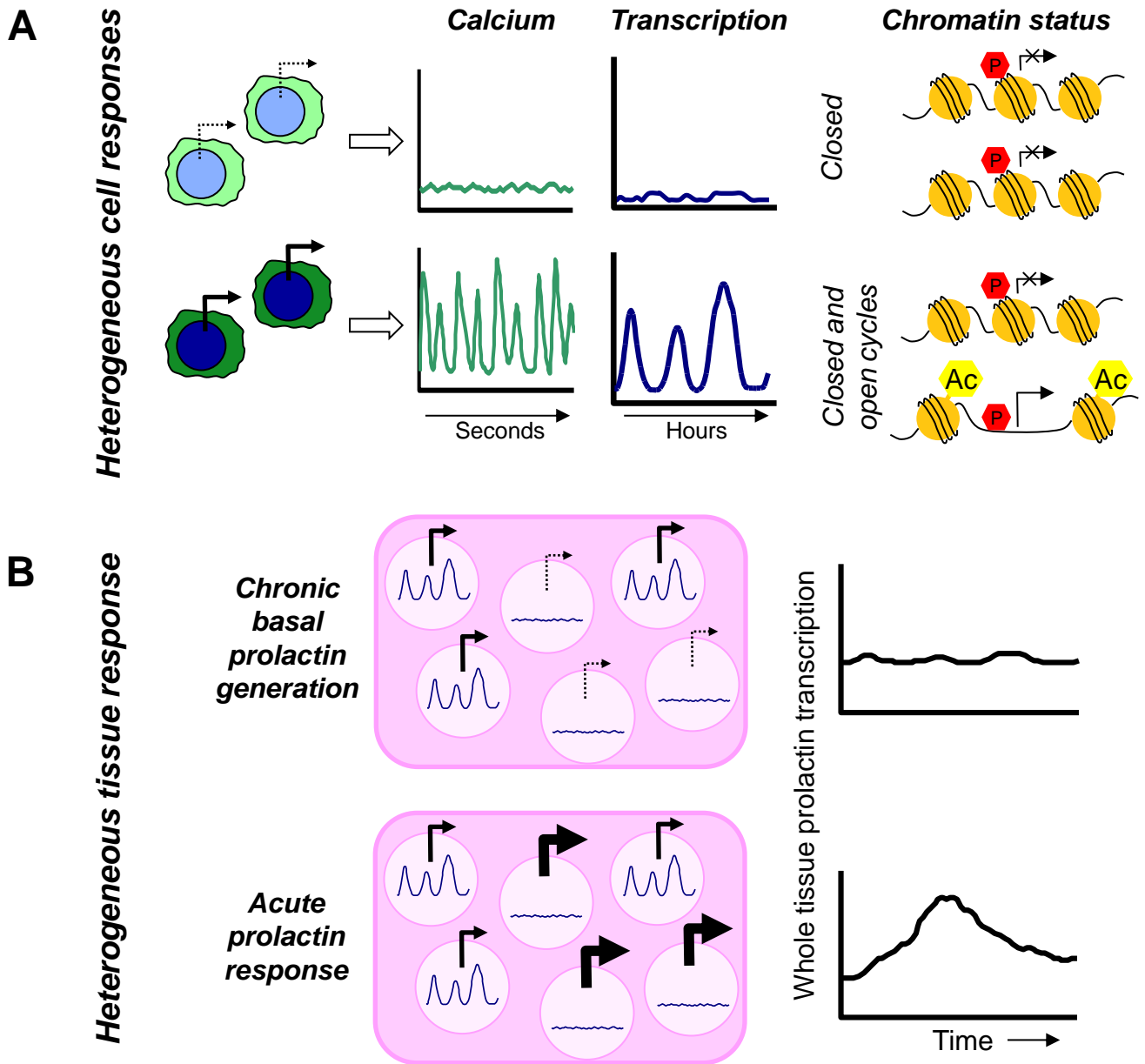


Figure 6

