The functional genomic response of developing embryonic submandibular glands to NF-kappaB inhibition
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Abstract

Background: The proper balance between epithelial cell proliferation, quiescence, and apoptosis during development is mediated by the specific temporal and spatial appearance of transcription factors, growth factors, cytokines, caspases, etc. Since our prior studies suggest the importance of transcription factor NF-κB during embryonic submandibular salivary gland (SMG) development, we attempted to delineate the emergent dynamics of a cognate signaling network by studying the molecular patterns and phenotypic outcomes of interrupted NF-κB signaling in embryonic SMG explants.

Results: SN50-mediated inhibition of NF-κB nuclear translocation in E15 SMG explants cultured for 2 days results in a highly significant increase in apoptosis and decrease in cell proliferation. Probabilistic Neural Network (PNN) analyses of transcriptomic and proteomic assays identify specific transcripts and proteins with altered expression that best discriminate control from SN50-treated SMGs. These include PCNA, GR, BMP1, BMP3b, Chk1, Caspase 6, E2F1, c-Raf, ERK1/2 and JNK-1, as well as several others of lesser importance. Increased expression of signaling pathway components is not necessarily probative of pathway activity; however, as confirmation we found a significant increase in activated (phosphorylated/cleaved) ERK 1/2, Caspase 3, and PARP in SN50-treated explants. This increased activity of proapoptotic (caspase3/PARP) and compensatory antiapoptotic (ERK1/2) pathways is consistent with the dramatic cell death seen in SN50-treated SMGs.

Conclusions: Our morphological and functional genomic analyses indicate that the primary and secondary effects of NF-κB-mediated transcription are critical to embryonic SMG developmental homeostasis. Relative to understanding complex genetic networks and organogenesis, our results illustrate the importance of evaluating the gene, protein, and activated protein expression of multiple components from multiple pathways within broad functional categories.

Background

Following a classic epithelial-mesenchymal interaction developmental program, the mouse neonatal submandibular salivary gland (SMG) is comprised of large and small ducts which terminate in lumen-containing, presumptive acini that express embryonic mucin [1–8].
Progressive prenatal morphogenesis begins as a solid outgrowth from the oral epithelium around E11.5, and is best conceptualized in stages [9]: Initial Bud, Pseudoglandular, Canalicular, and Terminal Bud. Epithelial cell proliferation is found in all stages, even after well-defined lumen formation in the Terminal Bud Stage. Epithelial cell apoptosis begins with the onset of lumen formation in the Canalicular Stage.

The proper balance between SMG epithelial cell proliferation, quiescence, and apoptosis is mediated by the appearance of transcription factors, growth factors, cytokines, caspases, etc. at specific times and places [10–14]. These SMG cellular and extracellular components may be visualized as a Connections Map which details the functional relationships within and between pathways (Fig. 1).

Complex networks of biological signaling pathways (Fig. 1) emerge from the interconnections of simple pathways under local control [15–17]. As such, these cellular pathways are more analogous to the mostly redundant, overlapping neural network of the brain than to traffic grids of intersecting streets and interacting vehicles. There are two general, not mutually exclusive, classes of interconnections: (1) junctions which serve as signal integrators and (2) nodes which split the signal and route them to multiple outputs [18]. Understanding the nonlinear dynamics of these interconnections is intrinsic to understanding the regulation of SMG morphogenesis. This requires the integration of transcriptomic, proteomic, phenomic, and bioinformatic approaches, not least because development, in its most basic sense, is genes plus context [19–22].

With the present experiments, we sought a glimpse of the extraordinarily complex behaviors of a focused signaling network (Fig. 1). To this end, we studied the molecular patterns and phenotypic outcomes of a nodal "short circuit", i.e., the inhibition of NF-κB activation and translocation to the nucleus to bind to NF-κB response genes. In most cell types, the NF-κB p50/p65 heterodimer is maintained as an inactive form in the cytoplasm bound to the inhibitory protein IκB. Exposure of cells to stimuli of NF-κB induces the rapid phosphorylation and subsequent degradation of IκB proteins. Released NF-κB dimers then translocate to the nucleus, bind to its cognate DNA elements, and induce the expression of target genes [23–25]. Activated, nuclear translocated, NF-κB transcription factor has been documented in the mouse embryo from the 1-cell stage onward [26,27]. Activated NF-κB translocation into the nucleus, directly or indirectly, effects the transcriptional control of over 150 target genes [28]. NF-κB enhances cell proliferation by stimulating the expression of cytokines such as TNF, IL-1, IL-2, IL-6, and IL-8, among others [28,29]; NF-κB inhibits apoptosis by inducing TRAF and cIAP expression which suppresses Caspase 8 activation [30], and by inhibition of p53 transactivation [31,32].

We interrupted the NF-κB signal in embryonic SMG explants using the cell-permeable peptide SN50, a potent inhibitor of NF-κB nuclear translocation [25,26], [33–35]. SN50-mediated inhibition of NF-κB nuclear translocation in SMG explants results in extensive apoptosis and a very substantial decline in cell proliferation. Functional genomic analyses demonstrate that inhibition of NF-κB signaling is associated with the altered expression of numerous components of the genetic network of related signaling pathways. This modified expression of genes and proteins associated with the inhibition of the cell cycle and the induction of apoptosis, as well as the increased activation of proapoptotic and compensatory antiapotptic pathways, provides a "snapshot" of the broad primary and secondary effects of NF-κB signaling during SMG development.

**Results and discussion**

NF-κB is well visualized in embryonic SMGs. In the Pseudoglandular Stage (~E14), NF-κB is primarily immunodetected in SMG branching epithelia, and, to a much lesser extent, in the mesenchyme (Fig. 2A). At the Canalicular Stage (~E15–16), NF-κB is primarily intronuclearized in the central regions of the terminal buds, and to a lesser extent, in the ductal cells facing the lumen (Fig. 2B, double arrows). By the Terminal Bud Stage (~E17–19), NF-κB is diffusely distributed throughout ductal and terminal bud epithelia (Fig. 2B, double arrowheads), with the intensity of immunostain being markedly diminished compared to the Canalicular Stage.

**NF-κB inhibition and SMG phenotype**

E15 SMG primordia were cultured for 2 days in the presence or absence of the cell-permeable peptide SN50, a potent inhibitor of NF-κB nuclear translocation [33–35]. SN50 is composed of a nuclear localization sequence (NLS) for NF-κB p50 linked to a cell-permeable carrier [33–35]. SN50 blocks the intracellular recognition mechanism for the NLS on NF-κB, thus inhibiting NF-κB’s translocation through the nuclear pore. After 2 days in culture, SN50-treated explants exhibit a substantial decrease in gland size and branching morphogenesis compared to controls (compare Fig. 3A to 3B). These 2-day SN50-treated explants demonstrate a highly significant 81% decline (t4 = 26.25; p < 0.001) in cell proliferation (Fig. 3A, B; Fig. 4A) and a significant 10-fold increase (t4 = 7.98; p < 0.001) in apoptosis (Fig. 3C, D; Fig. 4B). This substantial increase in apoptosis is associated with a highly significant > 4-fold increase (t4 = 22.66; p < 0.001) in activated (phosphorylated) p53.
Figure 1

Connections Map. This signaling map reflects the pathways investigated in SMGs. Known and putative connections are based on references [6], [11], [23], [36], [76]-[108].
To demonstrate that this SN50 phenotype is consequent to SN50-mediated inhibition of NF-κB nuclear translocation and not the nonspecific effect of exogenous peptide, we compared E15 + 2 SMG phenotypes in explants cultured in control media, 100 μg/ml SN50 peptide, or 100 μg/ml mutant SN50 (mSN50) peptide. As expected, we found a marked difference between control and SN50-treated SMGs but none between control and mutant peptide-treated explants (data not shown). In addition, since TNF/TNFR1 signaling has been shown to induce embryonic SMG cell proliferation and inhibit apoptosis in vitro [13] and TNF/TNFR1 signal transduction primarily signals by induction of NF-κB nuclear translocation [36], we postulated that TNF supplementation should have no inductive effect on SN50-treated SMGs.

(Fig. 5). This result is not surprising, given that NF-κB inactivates p53 [32].

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Thus, we cultured E15 control, SN50-treated, and mSN50-treated explants in the presence of TNF (rTNF, 10 U/ml) supplementation for 4 or more days. In this set of experiments, we extended the culture period to provide sufficient time to allow for possible TNF-mediated recovery. TNF supplementation induced NF-κB (p50 and p65) translocation (Fig. 6A), a marked increase in explant size, and a notable increase in cell proliferation (not shown); similar results were seen in TNF+ mSN50-treated explants (not shown). By contrast, NF-κB redistribution was not found in explants cultured in TNF + SN50 (Fig. 6B); rather, NF-κB was absent from epithelial cell nuclei and exhibited a very weak, diffuse cytoplasmic distribution. Moreover, TNF supplementation was unable to rescue the abnormal SN50 phenotype. Finally, the identical response of control and mSN50-treated SMG explants to TNF supplementation provides further evidence that this mutant peptide had no effect on NF-κB activation.

**Transcriptomic analysis**

To investigate transcriptional responses to NF-κB inhibition, we analyzed control and SN50-treated E15 + 2 SMG explants using cDNA arrays. Of the 1176 transcripts assayed on these arrays (including transcription factors, cell cycle regulators, growth factors, etc.), 691 (~60%) demonstrated a 1.5-fold or greater increase or decrease in expression with SN50-induced NF-κB inhibition (Fig. 7). Of these, we focused our attention on those signal transduction, cell cycle, and apoptosis transcripts related to the Connections Map (Fig. 1). With inhibition of NF-κB translocation into the nucleus, 53 Connections Map...
transcripts exhibit altered expression (Table 1). We used Probabilistic Neural Network (PNN) analyses to determine which transcript changes best discriminate control from SN50-treated explants. These analyses identified those transcripts with significant changes which are relatively more important in defining the SMG phenotype, regardless of the direction (up or down) of change.

Among the cell cycle transcripts with altered expression (Fig. 8A), PNN analysis shows that the increased expression of cyclin D2, p57, and Cdc25a, as well as decreased expression of PCNA, best discriminate control from SN50-treated explants. Cyclin D2, Cdc25a, and PCNA promote cell division; p57 inhibits cell division (Fig. 1). The significant decline in PCNA transcript (Table 1) is consistent with the highly significant (p < 0.001) decline in PCNA-defined cell proliferation (Fig. 4A).

Among apoptosis transcripts with altered expression (Fig. 8B), PNN analysis demonstrates that downregulated Caspase 1 transcript, almost alone, best discriminates control from SN50-treated explants. Caspase 1 activates Caspase 3 and appears to promote production of the cytokine IL-1β, which upregulates the transcription of both Caspases 1 and 3, additionally potentiating apoptosis [37,38]. Thus, this regulatory mechanism of caspase...
gene expression would likely be diminished in SN50-treated explants were they allowed to develop further in culture. Contemporaneous proteome analysis provides a very different profile (see Table 2 and text below).

Although many "Ras/Raf" growth factor pathway transcripts were upregulated (Table 1), as a group they were poor predictors of SMG phenotype (control v. SN50-treated). PNN analysis (Fig. 9A) shows that only IGF2, IGF2R, and IGFBP3 are best at discriminating control from SN50-treated explants.

TGF-β1 and TGF-β2 show a 2-fold increase (Table 1) which is not unexpected given that TGF-β and NF-κB are found to be inversely proportional to one another [39]. Nevertheless, among the TGF-β family transcripts and others related to their expression and signal transduction (Fig. 9B), BMP1, BMP3b, BMP8a, Smad7, and GR
best discriminate control from SN50-treated explants. BMPs inhibit cell proliferation via downstream Smad1/5/8 proteins whereas Smad7 inhibits TGF-β and activin signaling (Fig. 1). This inhibition of TGF-β/activin signaling is modulated through NF-κB-dependent inhibition of Smad7 [40]. In addition, there is a negative feedback between NF-κB and Smad7; activated NF-κB inhibits Smad7 promoter activity [41] whereas Smad7 inhibits NF-κB activation and potentiates apoptosis [42]. Curiously, the relative importance of increased Smad7 expression is 20 times greater than that of Smad1/5 vis. defining the NF-κB-inhibited explants. It is likely that, in the absence of NF-κB’s negative regulation, Smad7 signaling is upregulated, thereby sensitizing cells to apopto-
sis. Finally, the nearly 2-fold decrease in glucocorticoid receptor (GR) is also of high relative importance in defining the SN50-treated phenotype (Table 1). Glucocorticoids (CORT) function through the GR to both activate specific gene expression as well as transrepress NF-κB [41]. Since GR confers this latter effect by associating through protein-protein interactions with NF-κB bound at κB response elements [43–47], it is important to also evaluate changes in GR protein levels (see below).

Further, we utilized PNN analysis to determine the iterated composite relative importance among Connections Map (Fig. 1) transcripts which have altered expression as a consequence of inhibition of NF-κB translocation into the nucleus (Fig. 10). That is, we then subjected those transcripts with altered expression in each group (cell cycle, apoptosis, Ras/Raf, TGF-β family) previously shown in Figures 7 and 8 to be relatively important in defining the SN50 SMG phenotype to further PNN analysis. This transcriptomic analysis is a time-bound "snapshot" in which gene expression is indicative of possible future protein expression. It is instructive that, of the 53 Connections Map transcripts with altered expression (Table 1), 4 genes of diverse pathways but overlapping function best discriminate control from SN50-treated explants: PCNA, GR, BMP1, BMP3b. The declining PCNA and GR reflect the sharp decline in cell proliferation and branching; the increasing BMP1 and BMP3b similarly reflects inhibition of cell proliferation (Fig. 1).

| Function (Fig. 1) | Protein | Fold-Change | Function (Fig. 1) | Protein | Fold-Change |
|-------------------|---------|-------------|-------------------|---------|-------------|
| **Cell Cycle**    | PCNA    | 1.69↓       | **Cell Cycle**    | CyclinG1| 1.99↑       |
|                   | E2F3    | 1.86↑       |                   | CyclinG2| 2.06↑       |
|                   | CyclinA2| 1.89↑       |                   | Cdk4    | 1.86↑       |
|                   | CyclinB1| 2.13↑       |                   | Cdc25a  | 2.00↑       |
|                   | CyclinB2| 1.91↑       |                   | Bub1    | 1.58↓       |
|                   | CyclinD1| 1.64↑       |                   | Bub1b   | 2.33↑       |
|                   | CyclinD2| 2.45↑       |                   | wee1    | 2.11↑       |
|                   | CyclinE1| 1.64↑       |                   | p57     | 2.05↑       |
| **Apoptosis**     | p53     | 1.54↑       | **Apoptosis**     | Bad     | 2.00↓       |
|                   | Fas     | 1.79↑       |                   | Bax     | 1.82↑       |
|                   | FasL    | 3.17↑       |                   | Bcl2    | 1.58↑       |
|                   | FAF     | 2.01↑       |                   | Bclx    | 1.58↑       |
|                   | TRAIL   | 3.50↑       |                   | Caspase1| 2.03↑       |
|                   |         |             |                   | Caspase7| 1.90↑       |
| **Signal Transduction** | IGF1       | 1.83↑       | **Signal Transduction** | CREB1       | 1.91↑       |
|                   | IGF2    | 2.33↑       |                   | c-jun   | 2.49↑       |
|                   | IGF1R   | 1.53↑       |                   | c-myc   | 2.47↑       |
|                   | IGF2R   | 1.69↑       |                   | TGF-β1  | 2.03↑       |
|                   | IGFBP2  | 2.27↑       |                   | TGF-β2  | 2.06↑       |
|                   | IGFBP3  | 2.41↑       |                   | BMP1    | 2.19↑       |
|                   | IGFBP4  | 1.92↑       |                   | BMP3b   | 2.35↑       |
|                   | IGFBP5  | 1.81↑       |                   | BMP8a   | 1.67↓       |
|                   | IGFBP6  | 2.48↑       |                   | BMPR1b  | 1.92↓       |
|                   | FGFR1   | 2.93↑       |                   | BMPR2   | 1.87↑       |
|                   | FGFR4   | 1.67↓       |                   | Smad1   | 1.90↑       |
|                   | Ras     | 1.58↑       |                   | Smad5   | 1.50↑       |
|                   | B-Raf   | 2.01↑       |                   | Smad7   | 1.97↑       |
|                   | ERK6    | 2.02↑       |                   | GR      | 1.67↓       |

* This composite data represents the mean changes of 3 independent experiments.

Proteomic analysis
Our cDNA array analysis provides a good first approximation of likely protein differences. However, one cannot extrapolate from mRNA abundance to relevant protein levels [48]. A recent study by Aebersole and coworkers [48] analyzing yeast protein and mRNA abundance clearly showed that mRNA transcript levels are poor predictors of protein expression. They demonstrate
that some genes with comparable mRNA levels exhibited
a 20-fold difference in their protein expression while
mRNA levels of comparable protein expression varied as
much as 30-fold.

Thus, we next analyzed SN50-treated and control E15 +
2 SMG explants using 2-D Western Multiprotein Arrays
to determine protein differences. This technique allows
for the densitometric analysis of about 600 signal trans-
duction and other proteins simultaneously in each inde-
pendent sample (Fig. 11). As shown in Table 2, we find 18
proteins which have both a 1.5-fold or greater change
with NF-κB inhibition and are specifically related to the
Connections Map (Fig. 1). They include signal transduc-
tion, cell cycle, and apoptosis proteins that are either di-
rectly or indirectly downstream from activation of the
TNF, IL-6, EGF, IGF, and FGF signaling pathways. The

Figure 10
Iterated composite relative importance of all Connections Map
transcripts with altered expression. Transcripts previously
shown in Figures 8 and 9 to best discriminate control from
SN50-treated explants were subjected to further PNN analy-
sis to determine which transcripts are most discriminating in
defining the SN50-treated E15 + 2 phenotype. Refer to Table
1 for the direction and magnitude of change for each tran-
script.

Table 2: Proteins With Significant Changes In Expression After
Inhibition of NF-κB Nuclear Translocation

| Protein | Fold-Change | Function (Fig. 1) |
|---------|-------------|------------------|
| PCNA    | 3.7↓        | cell cycle       |
| E2F1    | 4.1↑        | cell cycle       |
| Chk1    | >5↑         | cell cycle       |
| Chk2    | >5↑         | cell cycle       |
| FADD    | >5↑         | apoptosis         |
| FAF     | 1.9↑        | apoptosis         |
| Caspase 6 | >5↑     | apoptosis         |
| PARP    | >5↑         | apoptosis         |
| Ras     | 1.9↑        | signal transduction |
| c-Raf   | >5↑         | signal transduction |
| Mek2    | >5↑         | signal transduction |
| ERK1    | 1.6↓        | signal transduction |
| ERK2    | 1.5↑        | signal transduction |
| Rsk     | 2.4↑        | signal transduction |
| JAK1    | >5↑         | signal transduction |
| STAT1   | 1.7↑        | signal transduction |
| JNK1    | >5↑         | signal transduction |
| GR      | 1.8↓        | signal transduction |

* This composite data represents the mean changes of 2 independent
samples.
significant decline in PCNA protein (Table 2) is consistent with the significant decline in PCNA transcript (Table 1) and PCNA-defined cell proliferation (Fig. 4A).

PNN analysis shows that among cell cycle proteins with altered expression (Fig. 12A), the increased expression of Chk1, Chk2, and E2F1 best discriminates control from SN50-treated explants. Of particular interest is E2F1. Among the five known mammalian E2Fs, the ability to induce apoptosis is unique to E2F1 [49]. Overexpression of E2F1 in several cell lines results in G2 arrest, as well as apoptosis via p53-dependent and p53-independent pathways [50–52]. The presence of a dysplastic SMG phenotype in E2f1+/− mice indicates that E2F1 plays an important role during SMG development [53]. Moreover, E2F1 overexpression in human salivary gland (HSG) cells diverted these cells into an apoptotic pathway [54].

Among apoptosis proteins with altered expression (Fig. 12B), PNN analysis demonstrates that increased expression of Fas, Caspase 6, and ERK 1/2 best discriminates control from SN50-treated explants. Caspase 6 is activated by active Caspase 3 and in turn cleaves lamin, resulting in nuclear membrane fragmentation [55]. FAF interacts with the cytoplasmic domain of the Fas receptor to potentiate Fas-mediated apoptosis [56,57]. Thus, the upregulated cell cycle inhibitors and apoptotic proteins clearly favor cell cycle arrest and death.

Among signal transduction proteins with altered expression (Fig. 12C), PNN analysis shows that members of all three growth factor pathways (Ras/Raf; JAK/STAT; JNK) have high relative importance in discriminating control from SN50-treated explants. Of particular note are c-Raf, ERK2, and JAK1. Raf plays a key role in the Ras signaling pathway (Fig. 1). That ERK2 is of very high relative importance is consistent with the observation that the MAPK/ERK overrides apoptotic signaling from Fas, TNF and TRAIL receptors [58]. It appears that effectors apart from the MAPK/ERK pathway may also mediate the anti-apoptotic function of c-Raf [55a]. Further, both the SHP-2/Ras and JAK/STAT3 pathways are activated by IL-6R/gp130 signaling (Fig. 1).

Moreover, it is especially noteworthy that the nearly 2-fold decline of glucocorticoid receptor (GR) (Table 2) is also of very high relative importance in defining SMGs deprived of NF-κB nuclear translocation. As noted above, CORT/GR binding both activates specific gene expression and transrepresses NF-κB [47]. To repress NF-κB, the GR associates through protein-protein interactions with NF-κB bound at κB response elements [44–47]. The precise relationship between decreased NF-κB-mediated transcription and a decreased GR protein expression is unclear.

Nevertheless, CORT/GR function is important to embryonic SMG morphogenesis [60]. Radioimmunoassays first detect SMG CORT in amounts >2 pg/gland on E15; Western analysis first detects SMG GR on E14 (0.14 fmol/gland). By E18, SMG CORT has increased more than 50-fold, and SMG GR has increased nearly 11-fold. The SMG GR function, as defined by its ability to bind a DNA response element (GRE). Increasing CORT/GR function in vivo is associated with a significant decline in TGF-β expression and a significant increase in cell division. SMG primordia cultured under serumless, chemically defined conditions, and deprived of CORT, exhibit a dramatic decline of SMG branching morphogenesis. It is reasonable, then, to assume that the high relative importance of diminished GR protein expression to the phenotype of SN50-treated SMGs is directly related to the significant (p < 0.001) decline in cell proliferation and branching (Fig. 3A, B; Fig. 4A).

The iterated composite relative importance of all Connections Map proteins with altered expression as a consequence of NF-κB inhibition was then determined (Fig. 13). This proteomic analysis is a time-based "snapshot" of proteins assumed to be associated with physiologic function at the moment of SMG harvesting. Viewing the most defining proteins with altered expression, it is clearly reflective of increased apoptosis (increased Chk1, Caspase 6, E2F1), decreased cell proliferation and branching (decreased GR), and, interestingly, increased expression of diverse signal transduction pathways (Ras/Raf/ERK, JNK) to compensate for the proapoptotic signal.

Analysis of activated pathway components
We then focused our attention on two particularly important pathways relative to cell proliferation and apoptosis, ERK 1/2 and Caspase 3. Downstream of activated ERK 1/2 is an upregulation of cell proliferation proteins and potentially enhanced cell division, as well as a protective effect over apoptotic signaling via suppressed activation of caspase effectors. Downstream of activated Caspase 3 are the sequellae of apoptosis, including PARP cleavage and inhibition of DNA repair, DNA fragmentation, and nuclear membrane fragmentation. The increase or decrease in expression of the components of any signaling pathway is not necessarily probative of pathway activity. Rather, it is the change in the level of activated protein that is physiologically important. Thus, we determined if SN50 treatment was associated with activation of the ERK1/2 and Caspase 3 pathways, using E15 + 2 control and SN50-treated explants, 1-D Western blot analysis, and antibodies specific to activated (phosphorylated/cleaved) proteins. Specifically, we evaluated the levels of activated c-Raf, ERK1/2, Caspase 3, and PARP using antibodies which identify only the phospho-
**Figure 12**
Relative importance of cell cycle, apoptosis, and signal transduction proteins with altered expression in defining control and SN50-treated phenotypes. These PNN analyses among cell cycle, apoptosis, or signal transduction proteins with altered expression identified which proteins best discriminate control from SN50-treated E15 + 2 explants. Refer to Table 2 for the direction and magnitude of change for each protein.
rylated or cleaved proteins and do not cross react with the inactive protein. We found a significant increase (p < 0.05) in activated ERK1/2, Caspase 3, and PARP in SN50-treated explants (Fig. 14); no change was seen in activated c-Raf levels between control and SN50-treated explants. The greater than 2-fold increase in Caspase 3 activation is associated with a 1.8-fold increase in PARP cleavage (Fig. 14) and a 10-fold increase in apoptosis (Fig. 4B). Since Caspase 3 is nodal to E2F1 (via p53), FAF (via Fas/Caspase 8), and Caspase 6 [33,36,37,39], our observation of increased activated Caspase 3 is consistent with the increased levels of E2F1, FAF, and Caspase 6 proteins (Table 2). Regarding the ERK1/2 pathway, we found a greater than 2-fold increase of activated ERK2 in SN50-treated glands (Fig. 13) associated with a 1.5-fold increase in total ERK2 protein (Table 2). This increased activity of proapoptotic (caspase3/PARP) and compensatory antiapoptotic (ERK1/2) pathways is consistent with the dramatic cell death seen in SN50-treated SMGs. Paradoxically, increased ERK1 activation is seen despite a 1.6-fold decrease in total ERK1 protein (Table 2) and increased ERK 1/2 activation is associated with virtually no change in the antecedent activation of c-Raf. The latter is consistent with the demonstration that c-Raf function is not mediated by the MAPK/ERK cascade [59]. Moreover, although we find an increase in total Raf protein (Table 2), no increase in activated c-Raf is found; we also see a 2-fold increase in activated Caspase 3 but no change in total Caspase 3 protein. These results clearly illustrate that changes in total protein level are not always indicative of altered protein activity.

Finally, it should be noted that a recent study using cell lines raised the possibility that SN50’s action is not specific to NF-κB [61,62]. SN50 is composed of the NLS for NF-κB p50 and was believed to specifically block NF-κB p50/p65 nuclear translocation by binding the NLS receptor complex and preventing transport through the nuclear pore [33–35]. However, Torgerson and coworkers [61] have shown that SN50 treatment inhibited nuclear transport of transcription factors NFAT, AP-1, STAT1, and NF-κB at a high dose of 210 µg/ml in Jurkat cells. However, others have shown that lower doses ≤100 µg/ml of SN50 specifically inhibited NF-κB nuclear translocation in human peripheral blood lymphocytes and murine T cells [33,63]. These reported differences are likely due to dose-dependent or cell-specific differences in the effect of SN50 [64]. Given that: (1) embryonic SMGs were cultured in the presence of 100 µg/ml SN50, (2) immunodetectable NF-κB was absent from SMG epithelia nuclei in TNF + SN50-treated explants, and (3) one cannot extrapolate observations in Jurkat cells to those in primary cells [64] or organ cultures, it is most probable that our observed interruption of SMG development is proximately due exclusively to the inhibi-
tion of NF-κB nuclear translocation. Indeed, for low doses of SN50, there is no evidence in the literature to the contrary. Nonetheless, we do recognize that absence of evidence is not necessarily evidence of absence.

Conclusions
Our results indicate that NF-κB-mediated transcription is directly or indirectly critical to embryonic SMG developmental homeostasis. We demonstrate the interplay between gene expression, protein expression, protein activity, and morphology in response to NF-κB inhibition. Gene/protein differences between control and NF-κB-inhibited phenotypes are not linearly causal of SMG dysplasia. In fact, these differences are discovered correlations between network components and an emerging SMG phenotype, a glimpse of nonlinear organogenesis [65].

Considering the outcome of this study relative to the Connections Map (Fig. 1), it is apparent that NF-κB nuclear translocation is functionally integral to a genetic network with broadly related, rather than independent, components. It may be said to represent the collective dynamics of a “small-world” network such that the average number of factors in the shortest chain connecting any two factors is small [66]. Such dynamical systems with small-world coupling display enhanced signal-propagation speed and synchronizability. Thus, if one focuses on the superimposition of the various layers of information, namely morphology, gene expression, protein expression, and protein activity (Figs. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14), one can visualize a coordinated, multidimensional response to inhibited NF-κB nuclear translocation. This visualization, however, is necessarily impressionistic even though our assays have some precision. This is so because we cannot extrapolate from transcriptome to proteome to activated proteins with any accuracy (in the absence of actual steady-state measures), and because in these experiments time is necessarily cross-sectional, not longitudinal. Nevertheless, relative to understanding a complex genetic network and organogenesis, our results demonstrate the importance of contemporaneously evaluating the gene, protein, and activated protein expression of multiple components from multiple pathways within broad functional categories. Understanding the signal dynamics of these pathways will require expanded models that encompass more aspects of regulation [e.g. [67]]. Still, we will always be limited by the fact that phenotypes are complex, emergent phenomena [16].

Materials and Methods
Tissue collection
Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as previously described [60]; plug day = day 0 of gestation. Pregnant females were anesthetized on days 15–19 of gestation (E15–18) with methoxyflurane (metofane) and euthanized by cervical dislocation. Embryos were dissected in cold phosphate buffered saline (PBS) and staged according to Theiler [68]. SMGs were dissected and cultured, processed for histology, or stored at -70°C. For cDNA expression and proteomic studies, E15 + 2 explants were collected, pooled, and stored at -70°C.

Culture system
E15 SMG (mostly Canalicular Stage) primordia were cultured using a modified Trowell method as previously described [13]. The medium consisted of BGJb (Life Technologies, Rockville, MD) supplemented with 0.5 mg ascorbic acid/ml and 50 units penicillin/streptomycin (Life Technologies), pH 7.2, and replicate cultures were changed every other day. Cultures were supplemented on day 0 and maintained for the duration of the experiments. In each of the enumerated studies, a minimum of 12 explants were cultured for 2 or 4 days in the cell permeable peptide SN50 (Biomol Research, Plymouth Meeting, PA) which inhibits NF-κB translocation into the nucleus [24, 33–35]. The concentration used (100 µg/ml) was double that shown to inhibit NF-κB translocation in mouse endothelial LE-II cells; 100 µg/ml mutant SN50 (mSN50) peptide was used as a positive control and control BGJb medium as a negative control. We evaluated their microanatomy by routine hematoxylin and eosin histology. We report a marked difference between SN50-treated and control explants or SN50 and mSN50 peptide-treated explants. No differences were observed between control and mSN50–treated explants. Since these initial studies demonstrated no difference between explants cultured in control media alone and in mutant peptide, control media was used as the control in all subsequent experiments. Ten independent experiments of E15 primordia were cultured for 2 days (E15 + 2) in CONT (control) or SN50-supplemented media, each group consisting of a minimum of 8 explants per group. E15 + 2 explants were collected and processed as described below.

To further demonstrate that SN50 treatment inhibited NB-κB activation, we evaluated if TNF supplementation would induce NF-κB translocation and SMG morphogenesis. E15 SMGs were cultured for 4 days or longer in 10 U/ml recombinant mouse TNF (rTNF, R & D, Minneapolis, MN), 100 µg/ml SN50 + 10 U/ml rTNF, or 100 µg/ml mSN50 + 10 U/ml rTNF, 6–10 explants per treatment group. This rTNF concentration was previously shown in our laboratory to induce embryonic SMG morphogenesis and cell proliferation [13]. Explants were collected and evaluated by histological and immunochemical analyses as described below.
Histology and immunolocalization
SMGs were fixed in Carnoy's fixative, processed, embedded in low-melting point paraffin, and stored for brief periods at 4°C as previously described [13]. Cultured explant morphogenesis was analyzed by dissecting microscopy and by light microscopy of serial sections stained with hematoxynlin and eosin. A minimum of 5 explants per group was evaluated for all experimental groups. For immunohistochemistry, the tissues were sectioned at 7 μm, placed on cleaned, gelatin-coated slides at 37°C for 3 hr, and immediately immunostained as previously described [9,13]. The sections were incubated in polyclonal goat anti-NF-κB p65/RelA antibody (C-20)(Santa Cruz Biotechnology, Santa Cruz, CA); this antibody has been shown to cross-react with mouse p65; it is not cross-reactive with RelB p68 or c-Rel p75. We confirmed the spatial distribution of NF-κB using a polyclonal goat anti-NF-κB p50 antibody (C-19) (Santa Cruz Biotechnology); this antibody has been shown to react with mouse p50 or p105; it is not cross-reactive with NF-κB p52, p65/RelA or p100. Controls consisted of sections incubated with preimmune serum or PBS alone. In this set of experiments, the cytoplasm appears blue and PCNA-positive nuclei appear dark brown. Quantitation of cell proliferation is presented as the ratio of PCNA-positive epithelial cells/total epithelial cells. Mean ratios per section and mean ratios per group were determined. Statistical comparisons were made between CONT and SN50-treated E15 + 2 explants as described below.

Apoptosis assay
Apoptotic cells were detected using a monoclonal antibody to single-stranded DNA (ssDNA) (Mab F7–26) according to the method of Apostain, Inc. (Miami, FL) [13]. Selective binding of anti-ssDNA monoclonal antibody F7–26 to apoptotic nuclei reflects decreased stability of DNA to thermal denaturation. Four positive and negative controls were conducted. Negative controls: (1) Tissue sections were heated and treated with S1 nuclease (Sigma); S1 nuclease eliminates staining of apoptotic cells, thus demonstrating that Mab F7–26 binds specifically to ssDNA. (2) Sections were pretreated in PBS containing lysine-rich histone (Sigma) prior to heating and immunostaining; reconstitution with histone restores DNA stability in apoptotic nuclei, thus preventing DNA denaturation and eliminating Mab staining of apoptotic cells. Positive controls: (1) Sections were heated in water and treated with Mab; bright staining of all non-apoptotic nuclei with low apoptotic indexes demonstrates that the procedure is adequate to detect ssDNA. (2) Sections were pretreated with proteinase K before heating; intensive staining of non-apoptotic nuclei demonstrates that the procedure detects decreased DNA stability induced by the digestion of nuclear proteins. Mab F7–26 was purchased from Apostain, Inc.

A apoptotic nuclei appear as dark brown. Since the sections were not counterstained with hematoxylin in this set of experiments, epithelial cell cytoplasm appears as light brown. Only apoptotic (variably intense dark brown) nuclei were counted in control and SN50-treated sections. Apoptosis was evaluated in a minimum of 4 explants per experimental group. Quantitation of apoptotic nuclei was conducted as described above for p53. Apoptosis is presented as the ratio of apoptotic-positive epithelial cell nuclei/total epithelial cell nuclei. Mean ratios per section and mean ratios per group were determined. Statistical comparisons were made between CONT and SN50-treated E15 + 2 explants as described below.

cDNA expression arrays
For cDNA Expression Array analysis, E15 SMG primordia were cultured in the presence or absence of SN50 peptide for 2 days (E15 + 2), collected in cold PBS containing 0.02% DEPC, snap frozen, and stored at -70°C. Clontech (Clontech Laboratories, Inc., Palo Alto, CA) Mouse 1.2 cDNA Expression Arrays were used to analyze each sample. These arrays include 1176 mouse cDNAs, 9 housekeeping control cDNAs, and negative controls im-
mobilized on a nylon membrane [www.clontech.com]. Briefly, total RNA was isolated and cDNA probes were synthesized using the Atlas Pure Total RNA Labeling System and 32P. The labeled cDNA probes were hybridized to the Atlas Array using ExpressHyb Solution. Hybridization signals were revealed by phosphorimaging and quantitated using the Clontech Atlas Image 1.01 software package, which allows for unbiased normalization of transcript abundance to overall signal. We generated pseudocolored images indicating up and down gene regulation. The probe set intensity (average difference) is proportional to the abundance of the specific mRNA it represents and was calculated by comparing hybridization signal of the control oligonucleotide to that of the treated. Total signal intensity of different probes was scaled to the same value before comparison. Fold changes were calculated by AtlasImage 1.0 software by pairwise comparisons of corresponding probe pairs from experimental and control. Three independent experiments were conducted per experimental group and the composite array determined. Relevant genes with altered expression were then assigned to functional groups. Specifically, we assigned those genes related to the Connections Map (Fig. 1) that have a 1.5 or greater fold-change to functional groups (i.e., cell cycle, apoptosis, signal transduction, etc.) which have biological significance.

2-D western array screening
The expression of signaling proteins was analyzed by Powerblot Western Array Screening (BD Transduction Laboratories, Lexington, KY). This 2-D Western Blot Array methodology simultaneously examines relative changes in protein expression in ~600 proteins in a given sample. Using highly specific monoclonal antibodies in antibody combinations carefully formulated by BD Transduction Laboratories, this multiprotein assay detects proteins to the nanogram levels and can distinguish closely related members of many important signaling families. E15+2 CONT and SN50-treated explants were collected and processed according to the protocol of BD Transduction Laboratories. Each sample (CONT and SN50-treated) was analyzed on 4 separate 2-D gels which were then transferred onto 4 blots. Each blot was then incubated with a different mixture of ~150 monoclonal antibodies and proteins were detected by chemiluminescence; ~600 (150 antibodies × 4 blots) proteins were evaluated in a given sample. For this set of experiments, two independent samples were analyzed. The relative level of proteins were determined by phosphor imaging and normalized to overall signal. We then assigned those Connections Map proteins with a 1.5 or greater fold-change to functional groups as described above.

1-D western blot analysis
To determine which key pathways were activated, Western blot analyses of phosphorylated or cleaved proteins in E15+2 CONT and SN50-treated explants were conducted as previously described [5]. For this set of experiments, we first determined the specificity for each of the following antibodies purchased from Cell Signaling Technology (Beverly, MA) using E15 and E17 SMG homogenates: anti-phosphorylated Erk1/2 [phospho-p44/42 MAP kinase (Thr202/Tyr204)] antibody, anti-phosphorylated c-Raf(Ser259) antibody, anti-cleaved Caspase 3 (D 175) antibody, and anti-cleaved PARP (D214) antibody. Each antibody had previously been shown to be specific for the activated (phosphorylated/cleaved) protein and not to cross react with inactive protein. Once optimal experimental conditions were established for each antibody, we then incubated blots of E15 and E17 SMGs in a mixture of these 4 antibodies and determined that we could identify all proteins in a single sample by Mn. This methodology using a mixture of antibodies has been successfully used by Cell Signaling Technology and BD Signal Transduction for 2-D and 1-D Western blot analyses. Controls consisted of blots incubated in preimmune rabbit serum or in the absence of primary antibodies; controls were routinely negative. In each sample, each activated protein was identified by Mn and the relative level of activated proteins in CONT and SN50-treated explants was determined by densitometry. The SN50 results are presented as fold change relative to CONT protein. Two independent samples per group was analyzed. Statistical comparisons were made between CONT and SN50-treated E15 + 2 explants as described below.

Probabilistic neural network analysis
We used PNN analyses to determine which Connection Map (Fig. 1) transcripts or proteins with altered expression best discriminate CONT from SN50-treated explants with 100% sensitivity and specificity [69]. PNN analyses identify the relative importance (0–1, with 0 being of no relative importance and 1 being relatively most important) of gene and protein expression changes in defining the SN50 phenotype. It is the change in expression, not the direction of change, that is important in defining the phenotype. The algorithm we used (Ward Systems Group, Frederick, MD) is based upon the work of Specht and colleagues [69–72]. Utilizing proprietary software designed by Ward Systems Group (Frederick, MD), we made comparisons among Connections Map transcripts or proteins with altered expression in a given group.

Statistical analysis
Means differences were analyzed by t-test in the usual manner [73]. To meet the assumptions of this analysis, namely normality and homoscedasticity (homogeneity...
of variances), counts, ratios, and percentages were log or arcsin transformed [74]. This allows for parametric statistical testing.

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