An NF-κB and Slug Regulatory Loop Active in Early Vertebrate Mesoderm

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Background. In both Drosophila and the mouse, the zinc finger transcription factor Snail is required for mesoderm formation; its vertebrate paralog Slug (Snai2) appears to be required for neural crest formation in the chick and the clawed frog Xenopus laevis. Both Snail and Slug act to induce epithelial to mesenchymal transition (EMT) and to suppress apoptosis. Methodology & Principle Findings. Morpholino-based loss of function studies indicate that Slug is required for the normal expression of both mesodermal and neural crest markers in X. laevis. Both phenotypes are rescued by injection of RNA encoding the anti-apoptotic protein Bcl-xL; Bcl-xL’s effects are dependent upon IκB kinase-mediated activation of the bipartite transcription factor NF-κB. NF-κB, in turn, directly up-regulates levels of Slug and Snail RNAs. Slug indirectly up-regulates levels of RNAs encoding the NF-κB subunit proteins RelA, Rel2, and Rel3, and directly down-regulates levels of the pro-apoptotic Caspase-9 RNA. Conclusions/Significance. These studies reveal a Slug/Snail–NF-κB regulatory circuit, analogous to that present in the early Drosophila embryo, active during mesodermal and neural crest formation in Xenopus. This is a regulatory interaction of significance both in development and in the course of inflammatory and metastatic disease.

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INTRODUCTION

The process of transforming relatively immotile epithelial cells into actively migrating mesenchymal cells, known as epithelium to mesenchymal transition (EMT), is central to a wide range of biological processes from mesoderm, mesenchyme, and neural crest formation to pathogenic fibrosis and metastasis [1–4]. Important players in the regulation of EMT are the zinc finger transcription factors Snail (Snai1) and its vertebrate paralog Slug (Snai2). In addition to Snail and Slug, a number of other members of the Snail family have been identified. In Drosophila melanogaster there are the Snail-like genes Escargot and Worniu [5–7], and the more divergent Scratch genes [8]. The duplication event that gave rise to Snail-like and Scratch-like genes appears to have occurred before the divergence of proteostomes and deuterostomes [9,10].

The involvement of Snail-like proteins in EMT was first suggested by genetic studies in Drosophila. Mutations in Snail lead to the disruption of mesoderm and embryonic lethality [11–13]. As in Drosophila, mice homozygous for a null mutation in the orthologous Snail gene fail to form normal mesoderm and exhibit early embryonic lethality [14]. No mesodermal phenotype was observed in mice homozygous for a null mutation in Slug [15,16]. The absence of Slug does lead to defects in melanocyte, hematopoietic stem cell and germ cell development, and epidermal healing [17–19]. Slug is expressed in the mesoderm in the chick and exposure of the early embryo to anti-sense oligonucleotides leads to defects in mesoderm emergence [20]. In X. laevis Slug mRNA is expressed zygotically in the dorsal mesendoderm; interference with its function, through the injection of RNAs encoding dominant negative proteins, leads to defects in the expression of organizer (Chordin, Cerberus) and ventrally (Xvent1, Xvent8) expressed genes [21]. An important concern about such studies involves the specificity of “anti-morphic” reagents, given the known regulatory cross-talk between E-box binding Snail, Slug and basic helix-loop-helix (bHLH) transcription factors (see below).

In both X. laevis and the chick, Slug appears to have an essential role in neural crest formation [20,22–24]. In contrast, mutation of Slug has no apparent effect on neural crest formation in the mouse [15]. This apparent discrepancy was initially ascribed to a swapping of Slug and Snail expression domains in the mouse [25,26]. More recent studies, using a combination of constitutive and conditional knock out mutations, indicate that neither Slug nor Snail are required for neural crest formation in the mouse, at least in the cranial region [16].

Snail-like proteins are generally thought to act as transcriptional repressors, although Sakai et al [27] report that Slug positively regulates own expression. Snail, Slug, and Scratch all bind to E-box sequences (CANNTG) and can antagonize the activity of bHLH proteins [8,28–31]. In their role as regulators of EMT, Slug and Snail have been found to suppress expression E-cadherin and tight junction components and the forced expression of Slug disrupts adherens junctions, tight junctions, and desmosomes [32–39]. Slug and Snail also act as inhibitors of apoptosis [40–44]. Slug has been found to negatively regulate the expression of the pro-apoptotic p53 [43] and Puma [45] genes. Subsequent studies have found that Slug is required for the metastasis of human melanoma cells [46] and has been implicated in lung adenocarcinoma and breast carcinoma invasiveness [47–49].
Sequence analysis indicate that Slugs are more conserved than vertebrate Snails [50]. Lespinet et al [10] grouped chick (Gallus gallus) and X. laevis Slugs with Slugs rather than with other vertebrate Snails. Slug and Snail have been found to be functionally similar, but not identical. For example, injection of RNA encoding Snail rescues the effects of anti-sense Slug RNA injection in X. laevis [22] and “Slug and Snail can be functionally equivalent when tested in overexpression studies” [23]. Over-expression of Snail leads to expansion of the neural crest domain in the chick, much as observed following over-expression of Slug [51,52]. On the other hand, the need for Snail expression in the early Drosophila embryo cannot be replaced by either Escargot or Worniu [53]. Snail and Slug differ in their ability to induce neural crest markers in X. laevis ectodermal explants [23], even though Slug alone has been found to rescue the effects on neural crest following the blocking of both Slug and Snail activity [see 24]. Slug appears to bind less strongly to regulatory regions in the E-cadherin protein than does Snail [38], while Slug, but not Snail, has been found to mediate genotoxic resistance in human mesothelioma cells [54]. A microarray-based analysis of MDCK epithelial cells found both common and distinct sets of genes regulated by Slug and Snail [55]. Given that Snail [56–59] and Slug [60] can be post-translationally regulated in terms of both stability and intracellular localization, it remains unclear whether the differences between the two proteins are intrinsic or are due to protein-specific post-translational effects.

Previous studies of Slug’s role in X. laevis have used either anti-sense RNA [22] or dominant-negative proteins [21,23,24,61,62] to disrupt Slug expression and/or activity. As part of a study to separate the role of Slug in EMT from its role as a regulator of apoptosis, we designed a modified anti-sense DNA oligonucleotide (a morpholino) that blocks Slug expression. In the course of analyzing the ability of the anti-apoptotic protein Bcl-xL to rescue the phenotypic effects of this morpholino, we uncovered an essential role for NF-kB as a regulator of Slug expression in the early embryo, a regulatory interaction analogous to that observed in the early Drosophila embryo, and not apparently described previously in a vertebrate.

RESULTS

Previous studies on the role of Slug in Xenopus have relied on injection of either anti-sense RNA directed against 3’ untranslated region of the SlugA mRNA [22] or RNAs encoding various dominant-negative proteins [21,23,24,61,62]. To complement these studies, we developed a morpholino (Slug MO) directed against the SlugA mRNA [22]. There are two Slug pseudoalleles in these studies, we developed a morpholino (Slug MO) directed against the SlugA mRNA [22] or RNAs encoding various dominant-negative proteins [21,23,24,61,62]. To complement the SlugA mRNA (FIG. 1A). The Slug MO blocked the in vitro translation of SlugA RNA that contained its target sequence but had no effect on the translation of mycGFP-Slug RNA, which lacks SlugA’s 5’ untranslated region (data not shown).

When injected into one cell of two-cell embryos, the Slug MO (10 ng/embryo) inhibited expression of the mesodermal markers Xbra (FIG. 1B,C), Xmes (FIG. 1D,E), and Antipodean (Apod)(FIG. 1F,G) in late blastula/early gastrula stage embryos. In neurula stage embryos, the Slug MO inhibited expression of Sox9 (FIG. 1H), a marker of cranial neural crest and otic placodes [64,65]. In later stage embryos, the Slug MO led to the loss of craniofacial cartilages and the otic vesicle (data not shown), very much as observed in embryos injected with Slug anti-sense RNA [22]. Injection of a control MO had no apparent effect on any of the markers examined (Table 1). As a control for the specificity of the Slug MO, embryos injected with Slug MO were injected with RNA encoding mycGFP-tagged Slug; both normal Sox9 expression (FIG. 1I) and craniofacial morphology (data not shown) were rescued; injection of RNA encoding myc-GFP did not rescue either phenotype (Table 1 and data not shown). Previously, we found that injection of Snail RNA rescued the phenotypic effects of anti-sense Slug RNA injection [22]. This is also the case with the Slug morpholino; injection of 500 pg/embryo Snail RNA rescued expression of both mesodermal and neural crest markers in Slug MO injected embryos (FIG. 1J–L; Table 1).

Maternal Slug RNA can be detected by RT-PCR [22 and data not shown] and is expressed zygotically in mesoderm [21]. The loss of Slug function during late blastula/early gastrula stages would be expected to influence both neural crest and placodal development, which depend upon signals from the mesoderm [46–70]. We generated plasmids that encode chimeric proteins consisting of glucocorticoid-binding regulatory domain [71,72] and either Slug alone (GR-Slug) or Slug linked to a C-terminal GFP moiety (GR-Slug-GFP). In the absence of dexamethasone, both GR-Slug proteins are inactive and had no apparent effect on the Slug MO’s ability to block Sox9 expression (Table 2). When Slug MO and GR-Slug RNA injected embryos were exposed to dexamethasone beginning at stage 11, Sox9 expression was efficiently recovered (Table 2).

To examine the timing of Slug’s role in neural crest formation, we compared the effects of activating the GR-Slug-GFP protein in mid-blastula (stage 8), early gastrula (stage 11), and late gastrula/early neurula (stage 13) embryos (FIG. 2; Table 2). Activation of Slug at stage 8 lead to a complete rescue of both mesodermal (Xbra) and neural crest/placodal (Sox9) marker expression. Efficient rescue of neural crest/placodal marker expression was also observed when Slug was activated at stage 11, but rescue was much less efficient when Slug was activated at stage 13 (FIG. 2; Table 2).

Bcl-2/Bcl-xL suppression of the Slug MO phenotype

Inhibition of Slug activity by injection of Slug MO (FIG. 3A), antisense RNA (data not shown) or RNA encoding a dominant negative version of Slug (ZnfSlug) leads to increased numbers of apoptotic cells as visualized by TUNEL staining, while injection of Slug RNA suppresses apoptosis [61]. In our studies, carried out at stage 16/17, the increase in TUNEL positive cells was most prominent outside of the neural crest stage Slug expression domain, and so presumably represent effects on earlier developmental events.

To distinguish Slug’s pro-EMT and anti-apoptotic activities, we injected embryos with RNAs encoding the anti-apoptotic proteins Bcl-2 (human) and Bcl-xL (X. laevis). Both had similar effects and observations using Bcl-xL are show here. As expected, injection of Bcl-xL RNA blocked the Slug MO induced increase in TUNEL staining (FIG. 3B, C). Bcl-xL also rescued the expression of the early mesodermal markers Apod (FIG. 3C,D) and Xbra (FIG. 3E,F), the neural crest/otic placode marker Sox9 (FIG. 3G,H; Table 1), and craniofacial morphology (data not shown). In stage 16/17 embryos, injection of Bcl-xL RNA led to a dramatic increase in the level and spatial extent of Slug expression (FIG. 3I). In ectodermal explants (animal caps), prepared at stage 8/9 from embryos injected with Bcl-xL RNA, and analyzed at stage 11 (~3 hours later), there was a small (~2x) but reproducible increase in the level of Slug mRNA, as determined by quantitative RT-PCR (FIG. 3J).
Bcl-xL and Slug regulate NF-κB activity

Bcl-2 and Bcl-xL are structurally similar cytoplasmic proteins. Bcl-2 can influence gene expression through effects on IκB kinase activity [IκK][73–77]. To examine Bcl-xL’s effects on NF-κB activity in Xenopus, we used the NF-κB responsive p3XκB-Luc reporter plasmid together a mutated form of human IκBa (IκBsa) and acetyl-11-keto-b-boswellic acid (AKBA). In IκBsa serines 32 and 36, normally phosphorylated by IκK, are mutated to alanines. The stable IκBsa polypeptide acts as a dominant repressor of NF-κB activity [78]. AKBA inhibits IκK activation [79–81]. Treating animal caps with 50 mM AKBA stabilized an epitope-tagged form of Xenopus IκBα (FIG. 4A). Fertilized eggs were injected with p3XκB-Luc and pTK-Renilla DNAs and Bcl-xL RNA, animal caps were prepared and then incubated in control media or in media containing AKBA. Bcl-xL increased NF-κB reporter activity and this increase was blocked by both IκBa and AKBA (FIG. 4B). On its own, AKBA had little effect on reporter activity.

Bcl-xL and Slug regulation of Rel expression

Five NF-κB subunit genes have been characterized in X. laevis: RelA/Rel1 [82,83], Rel2 [84], Rel3 [85], RelB [86] and Xp100 [87]. All five RNAs are supplied maternally and their levels drop at the onset of zygotic transcription (stage 8/9)(FIG. 5A). As development proceeds Rel2, Rel3 and Xp100 RNAs are again detectable by RT-PCR (28 cycles), while RelB RNA does not reappear until after stage 35 [86]. Bcl-xL RNA levels appear constant throughout this period [88](FIG. 5A). In stage 16/17 embryos, RelA, Rel2, Rel3, and Xp100 RNAs can be readily detected by RT-PCR in the anterior-dorsal quadrant of the embryo; the same region where Slug and Sox9 are normally expressed (FIG. 5B). RelA appears concentrated in the anterior dorsal sector, while Rel2 and Rel3, and to a lesser extend Xp100 appear to be present at similar levels throughout the embryo.

To explore the mechanism of Bcl-xL regulation of Slug and NF-κB, we generated a plasmid encoding a glucocorticoid-binding
Table 1. Slug Morpholino and Rescue experiments

| Injection                  | N   | Unaffected | Reduced/absent | In situ |
|---------------------------|-----|------------|----------------|--------|
| Slug MO                   | 42  | 2/42 (5%)  | 40/42 (95%)    | Sox9   |
|                           | 39  | 11/39 (28%)| 28/39 (72%)    |        |
|                           | 20  | 6/20 (30%) | 14/20 (70%)    |        |
|                           | 37  | 4/37 (10%) | 33/37 (92%)    |        |
|                           | 47  | 4/47 (8%)  | 43/47 (90%)    |        |
| Control MO                | 40  | 40/40 (100%)| 0/40 (0%)      | Sox9   |
|                           | 32  | 32 (100%)  | 0/32 (0%)      |        |
|                           | 26  | 26/26 (100%)| 0/26 (0%)      |        |
| Slug MO + mycGFP-Slug RNA | 45  | 39/45 (87%)| 6/45 (13%)     | Sox9   |
|                           | 42  | 37/42 (83%)| 5/42 (17%)     |        |
|                           | 23  | 20/23 (87%)| 3/23 (13%)     |        |
|                           | 43  | 28/43 (65%)| 15/43 (35%)    |        |
|                           | 30  | 5/30 (16%) | 25/30 (84%)    | Xbra   |
|                           | 27  | 2/27 (7%)  | 25/27 (93%)    | Xmenf  |
|                           | 22  | 4/22 (18%) | 18/22 (82%)    | Apod   |
|                           | 26  | 3/26 (12%) | 23/26 (88%)    |        |
| Slug MO + Snail RNA       | 56  | 53/56 (95%)| 3/56 (5%)      | Xbra   |
|                           | 28  | 25/28 (89%)| 3/28 (11%)     | Xmenf  |
|                           | 24  | 25/28 (89%)| 3/28 (11%)     | Apod   |
|                           | 46  | 42/46 (91%)| 4/46 (9%)      | Sox9   |
| Slug MO + hBcl2 RNA       | 28  | 24/24 (89%)| 2/8 (1%)       | Sox9   |
|                           | 15  | 14/15 (93%)| 1/15 (7%)      |        |
|                           | 41  | 27/41 (66%)| 14/41 (34%)    |        |
|                           | 49  | 32/49 (65%)| 17/49 (35%)    |        |
| Slug MO + Bcl-xL RNA      | 32  | 26/26 (81%)| 6/32 (19%)     | Sox9   |
|                           | 22  | 16/22 (72%)| 2/22 (28%)     |        |
| Slug MO                   | 31  | 8/31 (26%) | 43/31 (84%)    | Sox9   |
|                           | 33  | 6/33 (18%) | 27/33 (82%)    |        |
| Slug MO + RelA            | 40  | 23/40 (58%)| 17/40 (42%)    | Sox9   |
|                           | 86  | 65/86 (75%)| 21/86 (25%)    | Sox9   |
| Slug MO + Rel3            | 72  | 46/72 (64%)| 26/72 (36%)    | Sox9   |
|                           | 46  | 26/46 (56%)| 16/46 (44%)    |        |
| RelA ASP                  | 11  | 3/11 (27%) | 8/11 (72%)     | Slug   |
|                           | 26  | 8/26 (31%) | 18/26 (69%)    |        |
| Slug MO                   | 46  | 4/46 (9%)  | 42/46 (91%)    | Xbra   |
|                           | 35  | 26/35 (74%)| 9/35 (26%)     | Xbra   |
| Slug MO + Bcl-xL          | 29  | 20/40 (73%)| 11/40 (27%)    | Xbra   |
| RelA ASP                  | 28  | 6/28 (22%) | 22/28 (78%)    | Xbra   |
| IxBsa (500 pg/embryo)     | 32  | 9/32 (28%) | 23/32 (72%)    | Xbra   |
|                           | 16  | 5/16 (31%) | 11/16 (69%)    | Slug   |
|                           | 27  | 11/27 (41%)| 16/27 (59%)    | Sox9   |

*The Slug MO was injected at 10 ng/embryo into one cell of a two-cell embryo; mycGFP-Slug RNA was injected at 500 to 650 pg/embryo; Snail RNA was injected at 500 pg/embryo, Bcl-2, Bcl-xL, and RelA RNAs were injected at 600 pg/embryo, the RelA ASP was injected at 650 pg/embryo.

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A Vertebrate NF-κB-Slug Loop

To examine whether of NF-κB directly regulates Slug and Snail RNA levels, we first characterized the effects of RelA and RelA ASP in animal caps; injection of RelA RNA lead to an increase, while RelA ASP RNA lead to a decrease in Slug RNA levels (FIG. 7A). Using a dexamethasone-regulated form of RelA, GR-RelA, we found a similar effect – in the presence of domain-Bcl-xL-GFP (GR-BclxL-GFP) chimera. In the absence of dexamethasone, GR-BclxL-GFP does not alter Slug, RelA, Rel3 or Rel RNA levels, while addition of dexamethasone leads to their increase (FIG. 5C,D), similar to that seen using the non-hormone regulated form of Bcl-xL (FIG. 3J). No effect was observed on Xp100 RNA levels (data not shown). While these effects are small, i.e., 2–3 fold, they are highly reproducible.

Adding the protein synthesis inhibitor emetine blocks the dexamethasone-dependent increase in Slug and Rel2 RNA levels, but not the increase in RelA or Rel3 RNA levels; emetine alone had no reproducible effect on RNA levels (FIG. 5C,D). These results suggest that Bcl-xL acts directly to regulate RelA and Rel3, and indirectly to regulate Slug and Rel2 RNA levels. By “direct” we mean a regulatory interaction that does not require ongoing protein synthesis (see Discussion). Bcl-xL, activation also leads to an increase in the level of Snail RNA (FIG. 5E); this increase does not appear to involve effects on Slug RNA, since it occurs in the presence of the Slug morpholino.

AKBA treatment blocked Bcl-xL’s ability to increase levels of Slug, Snail and RelA RNAs (FIG. 5F), suggesting that Bcl-xL acts on these RNAs, as it does on the NF-κB responsive reporter, by increasing IkB kinase activity. RelAASP is a dominant negative form of RelA [89]; it dimerizes with other NF-κB subunit proteins and blocks their activity. When co-injected with RNAs encoding Rel3 or Xp52, the active form of the NF-κB subunit protein Xp100 [87], RelA ASP inhibited their ability to activate the 3Xusb-Luc reporter (FIG. 5G) and inhibited Bcl-xL’s ability to increase RelA and Slug RNA levels (FIG. 5H), indicating that active NF-κB is required for Bcl-xL to induce increases in Slug and RelA RNA levels.

Assuming that Bcl-xL acts to rescue the effects of the Slug MO through its ability to regulate NF-κB activity, injection of RelA RNA should be able to rescue the Slug MO phenotype. In embryos injected unilaterally with the Slug MO, injection of RelA RNA lead to re-appearance of both Xbra (FIG. 6A,B) and Apod (FIG. 6C,D) RNAs. In later stage, Slug MO-injected embryos, injection of RelA RNA lead to the reappearance of Sox9 expression in both the neural crest and otic placode regions, (FIG. 6E,F). A similar rescue of Sox9 expression in Slug MO injected embryos was observed upon injection of Rel3, or Xp52 RNAs (600 pg/embryo\(\text{Table 1}\)). We have not examined RelA’s effects on Slug RNA in Slug MO injected embryos because morpholinos typically stabilize, rather than induce the degradation of, their target RNAs (unpubl. obs.); RelA does induce an increase in Slug RNA levels, as monitored by \emph{in situ} hybridization, when injected on its own (data not shown – see below).

To complement these studies, we examined the effects of treating embryos with AKBA or injecting one cell of two cell embryos with either IxBsa or RelA ASP RNAs. AKBA treatment, beginning at the 4-cell stage, led to a noticeable decrease in the intensity of Xbra RNA staining in early gastrula stage embryos (FIG. 6G), but had little reproducible effect on Sox9 RNA levels in neurula stage embryos (data not shown). Injection of IxBsa RNA lead to the suppression of Xbra (FIG. 6H; \emph{Table 1}) and the reduction of Sox9 (FIG. 6I) expression. Injection of RelA ASP RNA inhibited expression of Xbra (FIG. 6J), Apod (FIG. 6K), and Sox9 (FIG. 6L).
dexamethasone GR-RelA produced an emetine-insensitive increase in Bcl-xL (FIG. 7B), Slug, and Snail RNAs (FIG. 7C). Given RelA’s ability to induce Sox9 expression in Slug MO injected embryos (see above), we examined RelA’s effect on Sox9 RNA levels; activation of RelA led to an increase Sox9 RNA levels even in the presence of emetine (FIG. 7C).

Table 2. Timing of Slug Rescue

| Injection * | Probe | Number of embryos | Percentage unaffected | Percentage reduced |
|-------------|-------|-------------------|-----------------------|--------------------|
| Slug MO+GR-Slug | Sox9 | 27 | 6/27 (22%) | 21/27 (78%) |
| +Dexamethasone | 43 | 35/43 (81%) | 8/43 (19%) |
| Slug MO+GR-Slug-GFP | Sox9 | 35 | 9/35 (25%) | 26/35 (75%) |
| +Dexamethasone at stage 11 | Sox9 | 85 | 77/85 (91%) | 8/85 (9%) |
| Activation time-course | Sox9 | 32 | 6/32 (18%) | 26/32 (82%) |
| +Dexamethasone at stage 8 | Sox9 | 36 | 30/36 (83%) | 6/36 (17%) |
| +Dexamethasone at stage 11 | Sox9 | 29 | 20/29 (68%) | 9/29 (32%) |
| +Dexamethasone at stage 13 | Sox9 | 31 | 8/31 (26%) | 23/31 (74%) |
| Xbra | 25 | 0/25 (0%) | 25/25 (100%) |
| +Dexamethasone at stage 8 | Xbra | 33 | 33/33 (100%) | 0/33 (0%) |

*One cell of a two-cell embryo was injected with SlugMO (10 ng/embryo) and GR-Slug-GFP (650 pg RNA/embryo). Ethanol-dissolved dexamethasone (20 μM) was added to the culture medium at stages 8,11,13; and maintained until the embryos were fixed at stage 10 for in situ hybridization analysis for Xbra or at stage 16/17 for Sox9.

**Targets of Slug regulation**

In Drosophila Dorsal, which encodes a RelA homolog, regulates Snail expression, and Snail in turn regulates Dorsal expression [see 90, 91]. In animal caps, the Slug MO decreased and mycGFP-Slug increased RelA RNA levels (FIG. 8A), suggesting an analogous

Figure 2. Timing of Slug rescue of Slug MO phenotypes: To analyze the timing of Slug activity in the early embryo, we injected one cell of two cell embryos with Slug MO (10 ng/embryo) together with RNA (650 pg/embryo) encoding the chimeric GR-Slug-GFP protein. A: In the absence of the activating drug dexamethasone, the Slug MO phenotype, i.e. suppression of Xbra expression in stage 11 embryos (A)(arrow) and suppression of Sox9 expression in stage 16 embryos (D), was unaltered. When embryos were treated with dexamethasone (20 μM) beginning at stage 8, there was an essentially complete rescue of Xbra (B,C) and Sox9 expression (E,H). Treatment of embryos with dexamethasone at stage 11 (early gastrulation) was also effective at rescuing Sox9 expression (F,H), while addition of dexamethasone at stage 13 (late gastrulation/early neurulation) produced at most a partial and inefficient rescue of Sox9 expression (G,H).

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regulatory circuit. To characterize Slug’s interactions with regulatory targets, we used the GR-Slug construct; its activity in the presence of dexamethasone is similar to that of mycGFP-tagged and untagged versions of Slug. In animal caps, both mycGFP-Slug and dexamethasone-activated GR-Slug lead to increased levels of the neural crest marker Zic5 [92,93] (data not shown) and Sox9 (FIG. 8B). In contrast Aybar et al., [23] reported that Slug did not induce neural crest markers in animal caps analyzed at stage 20, 22 hours after fertilization. To reconcile these observations, we analyzed animal caps derived from embryos injected with Slug RNA at stage 11 (our standard analysis point) and stage 16; Sox9 RNA levels were increased at stage 11 but had returned to control levels by stage 16 (FIG. 8C), indicating that factors in addition to Slug are required to maintain Sox9 expression. In independent studies we have found that levels of Sox3 and SoxD RNAs, whose expression is associated with early germ layer and neural differentiation, change dramatically in the period between stage 11 and 14 (C. Zhang, T. Grammer & M.W. Klymkowsky, unpubl. obs). GR-Slug positively but indirectly increased levels of Bcl-xL (FIG. 8D), Sox9 (FIG. 8E), RelA, Rel2, and Rel3 RNAs (FIG. 8F), but had no effect on Xp100 RNA levels (data not shown).

Figure 3. Rescue of Slug MO effects by Bcl-xL. A: Injection of the Slug MO leads to an increase in TUNEL staining. B: This increase is blocked by the injection of Bcl-xL RNA (600 pg/embryo and co-injected with LacZ RNA). Injected sides of embryos are marked by an "*" and red staining; line marks midline of the embryo, with anterior ("An") and posterior ("Ps") indicated. Injection of Bcl-xL RNA (600 pg/embryo) rescues Apod (C- Slug MO injected, D-Slug MO+Bcl-xL RNA injected), Xbra (E- Slug MO injected, F-Slug MO+Bcl-xL RNA injected), and Sox9 expression (G- Slug MO injected, H- Slug MO+Bcl-xL RNA injected). I: Injection of Bcl-xL RNA into one cell of a two-cell embryo led to a dramatic increase in the intensity and extent of Slug expression at stage 16; the region of Slug expression on the un.injected (control) side of the embryo is indicated by the dashed circle. J: Injection of Bcl-xL RNA produced an increase in Slug RNA levels in animal caps prepared at stage 8/9 and analyzed by QRT-PCR when uninjected embryos reached stage 11. Ornithine decarboxylase (ODC) was used as normalization control. RNA levels in the control case were set to 100%.
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Figure 4. Characterization of Bcl-xL effects on NF-κB activity. A: Fertilized eggs were injected with RNA (650 pg/embryo) encoding Xenopus IκBα-V5. Beginning at stage 8, experimental embryos were treated with 50 μM AKBA and analyzed at stage 11 by SDS-PAGE/immunoblot using an anti-V5 antibody and the antiSOX3c antibody to visualize endogenous Sox3 protein as a loading control. AKBA treatment stabilized the IκBα-V5 polypeptide. B: Fertilized eggs were injected with p3XκB-firefly luciferase ("3XκB-Luc") and pTK-Renilla luciferase ("RL-TK") DNAs (10 pg/embryo each) either alone ("Con") or together with Bcl-xL (500 pg/embryo) RNA, or Bcl-xL and IκBα (600 pg/embryo) RNAs. Alternatively, animal caps prepared from Bcl-xL RNA injected embryos were cultured in either control buffer (0.1% DMSO), 20 μM or 50 μM AKBA. At stage 11, caps were analyzed for luciferase activity. Bcl-xL induced an increase in 3XκB-Luc activity that was blocked by either IκBα or AKBA. Error bars in B reflect standard deviation from the mean of multiple experiments.
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Caspase-9 encodes an initiator caspase involved in the maternal/early embryonic apoptotic program in X. laevis, while the effector caspases-3 and -6 act downstream [94]. Caspase-9 appears to be a direct and negatively regulated target of Slug, while caspases-3 and -6 appear to be indirect targets (FIG. 8G). In embryos, Slug MO induced an increase in caspase activity as indicated by staining with the anti-activated caspase antibody CM1 and increased cleavage of a caspase-3 target peptide (data not shown).

These results extend those of Tríbulo et al., [61] and establish, apparently for the first time, a direct regulatory interaction between Slug and caspase-9.

DISCUSSION

In analogy with polymerization reactions, scientific studies often involve distinct initiation and catalytic events. In this work, the
Figure 6. NF-κB regulation of mesodermal and neural markers: The Slug MO induced loss of Xbra (A,B), Apod (C,D) and Sox9 (E,F) expression was rescued by injection of RelA RNA (600 pg/embryo)(A, C, E-Slug MO alone, B, D, F-Slug MO+RelA RNA). G: Treatment of early embryos with AKBA (50 μM from the 4-cell stage on) lead to a decrease in Xbra staining (control and AKBA-treated embryos marked). Injection of RNA encoding IκBsa (H,I) or RelAΔSP (J–L) had effects similar to that seen in Slug MO injected embryos; that is, both induced the reduction of Xbra (H,I), Apod (K) and Sox9 (L) RNA staining. AKBA treatment had no reproducible effect on Sox9 expression (data not shown). Arrows mark affected regions.

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Figure 7. NF-κB’s regulatory targets: A: In animal caps, RelA lead to an increase in Slug RNA levels, while RelAΔSP produced a decrease. When activated by dexamethasone (+Dex), the hormone-regulated form of RelA, GR-RelA (600 pg RNA/embryo), induced a similar increase in the levels of Slug RNA, as well as Snail, Sox9 (B), and Bcl-xL (C) RNAs compared to animal caps from GR-RelA injected embryos not exposed to dexamethasone. Similar effects were seen in the presence of emetine (+Dex+Eme), while emetine alone (+Eme) had little effect on any of measured RNA levels. Error bars in reflect standard deviation from the mean of multiple experiments.

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Figure 8. Slug’s regulatory targets: A: In animal caps analyzed at stage 11, the Slug MO (10 ng/embryo) produced a decrease in RelA RNA levels that was rescued by co-injection of mycGFP-Slug RNA (1 ng/embryo). B: Animal caps were prepared from embryos injected with either untagged or mycGFP-Slug RNAs; both produced a similar increase in Sox9 RNA levels. C: Animal caps, from embryos injected with either mt-GFP or mycGFP-Slug RNAs, were analyzed when control embryos reached stage 11 or stage 16; at stage 11 mycGFP-Slug induced an increase in Sox9 RNA levels, which returned to baseline by stage 16. No change in Sox9 RNA levels were observed at either stage in animal caps expressing mt-GFP. In GR-Slug injected caps, levels of Bcl-xL (D), Sox9 (E), RelA, Rel2 and Rel3 (F), and caspase-9, -3 and -6 (G) RNAs were increased in response to dexamethasone; with the sole exception of caspase-9, these increases were blocked by emetine. In all panels, error bars reflect standard deviation from the mean of multiple experiments.
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initiator was the observation that anti-apoptotic proteins rescue the effects of blocking Slug expression on mesodermal and neural crest markers. Bcl-xL produced an increase in both Slug and Snail RNA levels and Slug itself is sufficient to suppress the Slug morpholino phenotype (FIG. 1J-L)[22]. The role of catalyst was played by the observations of Kirshenbaum and colleagues [73–75,95], who found that Bcl-2 regulates NF-κB activity by activating IκB kinase (IκK). Activation of IκK induces the degradation of inhibitory IκB proteins, leading to increased NF-κB activity. Our studies using the dominant negative IκBα protein, the IκK inhibitor AKBA, and the dominant negative form of RelA, RelAΔSP, indicate that Bcl-xL regulates Slug RNA expression via NF-κB in the early X. laevis embryo (FIG. 9).

Mapping regulatory interactions

In mammalian epithelial cells over-expression of Bcl-2 has been found to promote EMT [96] and suppress cadherin expression [97]. Neither study, however, examined the effects of Bcl-2 expression on the levels of Slug or Snail RNAs. NF-κB itself has been implicated in EMT [98], and has been found to regulate Snail stability and activity through effects on glycosynthes kinase 3 [99]. In X. laevis, few promoters have been rigorously defined. It is possible, however, to tentatively classify regulatory interactions as direct or indirect based on the ability of hormone-regulated proteins to influence target RNAs in the presence of protein synthesis inhibitors. If a regulatory interaction requires or depends upon on-going protein synthesis, it is classified as “indirect”; “direct” interaction are not blocked by protein synthesis inhibitors.

Regulatory interactions are often complex and multifaceted; it is important to remember that conclusions based on hormone-regulated proteins need to be characterized further. For example, if a transcription factor regulates the expression of a gene encoding a microRNA, which in turn regulates the stability of a target RNA, its effects will appear as direct even though they are mechanistically indirect. As another example, NF-κB acts as both a transcriptional regulator [100] and has been reported to destabilize certain RNAs [101]. This latter activity would appear as direct in our system. In this light it is interesting to note that activation of GR-RelA leads to a protein-synthesis independent decrease in levels of p53 RNA (unpubl. obs.); whether this reflects transcriptional, post-transcriptional, or microRNA-mediated regulation is not yet resolved.

Neither Bcl-2 or Bcl-xL are thought to regulate gene expression through direct interactions with DNA or transcription factors, but rather through effects on various kinases [see 74–76]. Using a hormone-regulated form of Bcl-xL, RelA and Rel3 appear to be direct, while Rel2 and Slug appear to be indirect targets of Bcl-xL regulation. The ability of Bcl-xL to induce changes in RelA, Slug and Snail RNA levels is inhibited by both the dominant negative form of RelA, RelAΔSP and the IκK inhibitor AKBA, suggesting that Bcl-xL activates IκK, which initiates the destruction of IκB polypeptides, leading to the activation of pre-existing NF-κB, which in turn regulates target genes (FIG. 9). The presence of RelAΔSP or AKBA blocks these NF-κB-dependent processes and so blocks the effects of Bcl-xL on both “direct” and “indirect” targets.

NF-κB regulation of Slug

NF-κB subunit proteins are expressed ubiquitously and play a key role in cellular inflammation and tumor progression [102–106]. NF-κB is known to have a number of regulatory targets, including IκBα [107,108], which acts to repress, and so limit, NF-κB activity. In mammalian systems, NF-κB regulates the expression of a range of anti-apoptotic proteins, including the anti-apoptotic caspase inhibitor proteins (IAPs), Bcl-2, and Bcl-xL [109–113] and decreases the activity of the pro-apoptotic p53 protein in renal cell carcinoma cells [114]. In addition, NF-κB and p53 can inhibit each other’s activities by competing for the limited pool of CBP/p300 within the cell [115]. In X. laevis RelA/NF-κB is a positive regulator of Bcl-xL, Slug and Snail. Slug’s ability to down-regulate the pro-apoptotic genes Caspase-9 [61] and Puma [45] would be expected to generate an over-all anti-apoptotic state.

In this light, the decrease in NF-κB RNA levels at the midblastula transition (FIG. 5A) may be permissive in the regulation apoptotic processes in later stage embryos [116–118]. A number of drugs, e.g. AKBA and curcumin [79,81,119–121], inhibit NF-κB activity and increase apoptosis, perhaps by reducing levels of Slug and/or Snail expression, which may explain at least part of their anti-tumor effects.

Slug regulation of NF-κB

In X. laevis Slug activates an NF-κB responsive reporter and acts indirectly to increase levels of RelA, Rel2, and Rel3 RNAs. In Drosophila Snail also acts indirectly to regulate Dorsal (RelA) by inhibiting expression of WntD, which acts to inhibit activation of Dorsal [90,91]. How Slug regulates RelA/Rel3 expression in Xenopus remains unclear, but preliminary studies indicate that Drosophila WntD, as well as a number of Xenopus Wnts, inhibit Bcl-xL-mediated activation of the 3XκB-Luc reporter and reduce RelA RNA levels (Zhang & Klymkowsky, unpubl. obs.). Whether Slug acts through the regulation of a Wnt or some other intermediate, it is apparent that Slug can increase NF-κB activity and RNA levels in the early Xenopus embryo. Our studies indicate that NF-κB regulates both Slug and Snail RNA levels and plays an essential role in mesoderm formation. The presence of a heretofore unrecognized NF-κB-Slug/Snail regulatory loop in a vertebrate should have important consequences for our understanding the conserved and divergent evolutionary mechanisms involved in germ layer specification, as well as practical implications for therapeutic interventions that target NF-κB and Slug/Snail-mediated EMT and anti-apoptotic processes.

MATERIALS AND METHODS

Embryos and animal caps

X. laevis embryos were obtained following standard protocols [22,72] from adult animals purchased from Xenopus I, Inc. (Dexter, MI). Embryos were staged according to Nieuwkoop and Faber [122]. Fertilized eggs or one-cell of two-cell embryos were injected with 10–20 nL of solution; ectodermal explants (animal caps) were prepared from stage 8/9 embryos using a Gastro-
master™ (Xenotech) and cultured until control embryos reached either stage 11 or stage 16/17 [72,123]. In experiments involving hormone activation of chimeric polypeptides, whole embryos or animal caps were treated with 20 μM dexamethasone (Sigma) alone, or were pretreated for 30 minutes with 100 μg/mL of the protein synthesis inhibitor emetine (Sigma) [124], prior to dexamethasone and emetine treatment. In contrast to cycloheximide [125], emetine does not induce nodal gene expression under these conditions [126,127]. RNA was isolated and subjected to either standard or quantitative RT-PCR (QRT-PCR) analysis as described previously [123]. Primers for PCR analyses were:
caspase-3 [5′AAGTCGGACATCGAGGAGC3′; 5′TAAATTGAGCCCCCTCTAC3′]; 
caspase-6 [5′TGGACATCAAGACTGTTGAC3′; 5′CTGCGAACATCAACCCGAGG3′]; 
caspase-9 [5′CCGATGAGTTTCAAGCAA3′; 5′GACGGCAGAACGAGTTTC3′]; 
Bcl-xL/Xt1 [5′GTCGGGCTGTATGGAAAGA3′; 5′C-GATATGCGGACACGGATG3′]; 
Bcl-xI [5′GAAATGCAAGACTGTTCC3′; 5′TCTAGGGCAAGAATTGCTC3′]; 
Snail [5′AAGCAGAATGCGACTCTT3′; 5′CCTAATGTATGACACACCC3′ [22]]; 
Sox9 [5′GAGAATGTGAGGACGGACACCTGGC3′; 5′CTGGTGCGTGTGTCACGTGTAATG3′] [this study];
RelA [5′GGCGACTCGGAGGCTGCTGGTAGAAGGC3′; 5′GGGAATTTCTTTCATGCCCAAGCAC3′]; 
RelB [5′GAGCTTCCATCAAGCTAAAC3′; 5′GGTGTCGGTACGCTTGGTC3′];
RelF [5′ATCGAGAGGTTTGGACC3′; 5′GGGGTG-GTGAATATGTTGTA3′];
Sox10 [5′GCCATTGTACTGAACTGGC3′; 5′GCCAG-TCATTACCTCAGCAAGCC3′];
Xpl10 [5′CTGTAAGAATGCGAGATTAC3′; 5′GCACATGAGGTCTACTGAC3′] [this study].

Plasmids, morpholinos, and reporters
pCS2mveGFPSnug and pCS2mt-GFP have been described previously [22]. Plasmids encoding dexamethasone-regulated versions of Snug, RelA and Bcl-xL were generated by subcloning into the pCS2GR-Sox9-GFP plasmid [127]. A plasmid encoding an epitope-tagged form of Xenopus Bcl-xL (Xt1) [88] was supplied by James Maller (UCHSC, Denver, CO); the pCS2-LacZ plasmid epitope-tagged form of Xenopus Bcl-xL (Xr11) was supplied by Hugh Woodland (U. Warwick) [83]. Beak et al. 1998), Ken Kao (Columbia University); plasmids encoding Xenopus IκBsa is resistant to IκB-Slug Loop

TUNEL, anti-caspase staining and caspase cleavage assays
Fixed and sectioned embryos [134] were stained by TdT-mediated dUTP-biotin nick end-labeling (TUNEL) using a peroxidase-based kit purchased from Molecular Probes, following the manufacturer’s instructions. Whole-mount TUNEL [135] was carried out using the protocol on the Harland Lab website. The rabbit anti-activated caspase 3 antibody CM1 (BD Bioscience Pharmingen) was used in whole-mount immunocytochemistry at a dilution of 1:1000 following standard immunocytochemical techniques [136,137]. For caspase cleavage assays, embryo lysates were prepared and reactions were carried out in duplicate using one embryo equivalent of lystate (20 μL) and 80 μL lysis buffer. Reactions were incubated at 37°C for 1 hour with 5 μM of the caspase-3 fluorogenic substrate Ac-DEVD-AMC (BioMol), after which 990 μL of water was added and fluorescence was measured using a Hitachi F2000 Fluorescence Spectrophotometer. Results were analyzed for statistical significance using Student’s t-test of the means.

In situ hybridization and Alcian Blue Staining
Plasmids containing the Sox9 coding sequence, isolated by RT-PCR from neural stage embryos (Fawcett & Klymkowsky, unpublished). Digoxigenin-labeled anti-sense probes were generated against Sox9, Slug [70], Sox2 [130], Sox3 [139], epidermal keratin [140], Xbra [141], Antipodean (Apop) [142], and Xmenf1 [143] RNAs and in situ hybridization was performed following standard protocols [22,72]. Alcian Blue staining was carried out as described previously [134]. Digital images were captured using a Nikon CoolPix 995 Camera on an Inverted Leica M1400 Photomicroskop. Images were manipulated with Fireworks 8 software (Macromedia now Adobe) using the “auto levels” and “curves” functions only.

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This manuscript is dedicated to Martin Raff, a constant inspiration and a friend to many.

Author Contributions
Conceived and designed the experiments: MK CZ TC. Performed the experiments: MK CZ TC ET. Analyzed the data: MK CZ TC ET. Contributed reagents/materials/analysis tools: TS. Wrote the paper: MK CZ.

1 http://tropicalis.berkeley.edu/home/gene_expression/TUNEL/TUNEL.html
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