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Accessibility
Heat shock-inducible Cre/Lox approaches to induce diverse types of tumors and hyperplasia in transgenic zebrafish

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RAS family members are among the most frequently mutated oncogenes in human cancers. Given the utility of zebrafish in both chemical and genetic screens, developing RAS-induced cancer models will make large-scale screens possible to understand further the molecular mechanisms underlying malignancy. We developed a heat shock-inducible Cre/Lox-mediated transgenic approach in which activated human kRASG12D can be conditionally induced within transgenic animals by heat shock treatment. Specifically, double transgenic fish Tg(B-actin-LoxP-EGFP-LoxP-kRASG12D; hsp70-Cre) developed four types of tumors and hyperplasia after heat shock of whole zebrafish embryos, including rhabdomyosarcoma, myeloproliferative disorder, intestinal hyperplasia, and malignant peripheral nerve sheath tumor. Using ex vivo heat shock and transplantation of whole kidney marrow cells from double transgenic animals, we were able to generate specifically kRASG12D-induced myeloproliferative disorder in recipient fish. This heat shock-inducible recombination approach allowed for the generation of multiple types of RAS-induced tumors and hyperplasia without characterizing tissue-specific promoters. Moreover, these tumors and hyperplasia closely resemble human diseases at both the morphologic and molecular levels.

Results

Human kRASG12D Expression Can Be Induced in Transgenic Zebrafish by Heat Shock-Induced Cre-Mediated Recombination.

To express the human kRASG12D transgene in various tissue types, a 2.7-kb zebrafish B-actin promoter was used to drive the expression of a floxed transgene cassette containing an enhanced green fluorescent protein (EGFP) followed by a strong transcriptional stop element. This floxed EGFP cassette lies upstream from a constitutively active human kRASG12D transgene (Fig. 1A). Ten stable transgenic zebrafish B-actin-LoxP-EGFP-pA-LoxP-kRASG12D lines were generated (LGL-RAS). All stable lines expressed EGFP as embryos, whereas only four lines had broad EGFP expression as adults (17), suggesting that transgene expression is affected by positional effects (for line 25A, see supporting information (SI) Table 2 and SI Fig. 6). A heat shock-inducible Cre line (hsp70-Cre) was obtained (generously provided by Andre Quinkertz and the late Jose Campos-Ortega, University of Cologne, Cologne, Germany), and Cre expression was optimized by varying the stage and intensity of heat shock.

Abbreviations: LGL-RAS, B-actin-LoxP-EGFP-LoxP-kRASG12D; MPD, myeloproliferative disorder; MPNST, malignant peripheral nerve sheath tumor; RMS, rhabdomyosarcoma; dpf, days postfertilization; hpf, hours postfertilization.

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duration of heat shock at 37°C (SI Fig. 7). In brief, heat shock for 30–120 min at 37°C leads to optimal Cre expression in hsp70-Cre transgenic embryos, and treatment from either 4–5 hours postfertilization (hpf) or 24–25 hpf results in a high level of recombination at the genomic DNA level (Fig. 1J and SI Fig. 8). When individual double transgenic embryos (LGL-RAS, line 25A; hsp70-Cre) were heat shocked from 4 to 5 hpf, recombination at genomic level could already be detected 4 h after heat shock (8 hpf, 23.2 ± 8.5% of the transgenes recombined; n = 8 animals), whereas, non-heat-shocked animals did not exhibit recombination (0.3 ± 0.4%; n = 7). By 24 hpf, 32.6 ± 12.2% of the transgenes had recombined in the heat shock cohort (n = 8), whereas non-heat-shocked animals exhibited significantly less recombination (5.3 ± 2.5%, P < 0.001; n = 8). kRASG12D transcripts were first detected by whole-mount in situ hybridization at 16 hpf with heat shock-treated animals having 123 ± 25 kRAS-positive cells per animal (n = 12 animals), whereas expression was absent in non-heat-shocked animals (0.4 ± 0.3 cells per animal, n = 12; Fig. 1K and SI Fig. 9). Consistent with the low-level genomic DNA recombination detected in non-heat-shocked embryos at 24 hpf, these animals exhibited significantly less but detectable kRASG12D expression compared with heat-shocked siblings (129 ± 30 kRAS-positive cells per animal in the heat-shocked group, n = 7 vs. 1.3 ± 0.9 in non-heat-shocked group, P < 0.001; n = 4, Fig. 1G and K).

**Early Activation of kRASG12D Leads to Juvenile Lethality and Four Types of Tumors and Hyperplasia in Adult Transgenic Fish.** A cohort of 300 double transgenic embryos (LGL-RAS; hsp70-Cre) was analyzed to determine whether somatic activation of kRASG12D would lead to tumor formation (21). One hundred eighty animals were heat shocked at 24 hpf for 1 h at 37°C, whereas 120 nontreated animals served as controls. Heat shock-treated, double transgenic animals were smaller in body size (by 43 days postfertilization (dpf), average body length = 0.945 ± 0.367 cm (n = 8) compared with 1.89 ± 0.09 cm in the control group (n = 12), P < 0.0001 and displayed a significant survival disadvantage (P < 0.0001, log rank analysis) (Fig. 2A). To investigate the reasons for juvenile lethality in heat-shocked animals, a second cohort of double transgenic
animals was heat shocked and analyzed at 15, 20, and 25 days after fertilization. *In situ* staining of paraffin-embedded sections revealed that *kRASG12D* was expressed in multiple types of tissue, including intestinal epithelium (35–62% of juvenile fish at the age of 15–25 days), muscle (18–26%), neural cells (6–13%), and kidney marrow (0–9%) (SI Fig. 10). Among them, the intestinal epithelium is the most prominently affected tissue type, suggesting that gastrointestinal dysfunction may lead to stunted growth and juvenile death in the heat-shocked animals.

**Double transgenic animals** surviving past 25 days developed external tumor masses, difficulties in breathing and swimming, and/or progressive paleness. Twenty-five heat-shocked animals exhibiting such symptoms (average latency, 34.9 ± 6.2 days) were killed for pathological examination. In contrast, non-heat-shocked animals developed similar symptoms with a longer latency (n = 19, average latency, 65.8 ± 21.3 days; P < 0.01). *LGL-RAS* or *hsp70-Cre* single transgenic animals that received heat shock did not develop signs of disease by 6 months of age (n = 60 fish per line). Histological examination of double transgenic diseased fish revealed four different types of malignancy, including skeletal muscle tumors (RMS), MPD, intestinal epithelial hyperplasia, and in rare cases, malignant peripheral nerve sheath tumors (MPNST) (SI Table 3). All four abnormalities were observed in both groups, but incidence and latency differed between heat-shocked and non-treated animals (Fig. 2B). The malignancies observed in the non-heat-shocked animals were likely induced by aberrant activation of the *hsp70* promoter and subsequently Cre-mediated RAS activation.

The most common tumor type was RMS, with 76% tumor-bearing fish having skeletal muscle tumors in the heat-shocked group (Fig. 3B). RMS comprised small, round blue cells interpersed with atypical terminally differentiated striated muscle fiber cells (Fig. 3G). RNA *in situ* hybridization detected *kRASG12D* expression in the perinuclear area of the multinucleated striated muscle fiber cells and in the mononuclear tumor cells (Fig. 3H). Tumors identified histologically as RMS expressed clinical diagnostic markers of human RMS, including *desmin*, *myoD*, and *myogenin* (Fig. 3 I and J and data not shown). In contrast, only 37% of non-heat-shocked tumor-bearing fish developed RMS. RMS family members are mutationally activated in 5–35% of embryonal rhabdomyosarcomas in human (22), suggesting that RAS pathway activation is required for initiation of this disease in both zebrafish and humans.

The second most common lesion was MPD (Fig. 4B). Heat-shocked fish developed this disease with a latency of 34 days (n = 2 of 25 fish), whereas non-heat-shocked fish developed MPD with a prolonged onset (mean latency = 66.2 ± 23.1 days, n = 10 of 19 fish; SI Table 3). Histological analysis revealed an expansion of hematopoietic cells in kidneys, comprising predominantly myeloid cells in various differentiation stages (Fig. 4G). *In situ* staining confirmed the expression of *kRASG12D* in a subset of kidney marrow cells (Fig. 4H). *L-plastin*-expressing monocyte/macrophage cells (23) and *mpo*-expressing granulocytes (24) were increased in diseased fish compared with single transgenic *LGL-RAS* animals (Fig. 4 I and J), confirming the expansion of the granulocyte and monocyte/macrophage compartments.

To characterize the MPD arising in these animals further, kidney marrow cells were collected and assessed for total cell number, flow cytometric analysis, and differential cell count. Diseased fish had increased numbers of total hematopoietic cells in the kidneys compared with age-matched, single transgenic *LGL-RAS* fish (3.16 × 10⁶ ± 1.28 × 10⁶ vs. 9.45 × 10⁵ ± 2.31 × 10⁵ cells; n = 4 per group, P = 0.014). Flow cytometric analysis of MPD kidney marrow cells (25) revealed a distinct population of cells lying between myeloid and progenitor populations (Fig. 4M). MPD-affected fish also had severely decreased numbers of mature red blood cells, whereas lymphoid populations remained largely intact. Differential cell counts performed on cytopsin of the whole kidney marrow cells (Fig. 4N and Table 1) showed an increase in differentiated myelomonocytes (53.8% ± 4.6% vs. 28.8% ± 4.1%; n = 4 per group, P = 0.0002) and myeloid precursors (14.8% ± 3.3% vs. 6.2% ± 1.3%; P = 0.003) in diseased animals. Erythroid cell development was also perturbed with mature red blood cell numbers in the kidney being severely decreased compared with control animals (7.3% ± 5.6% vs. 41.3% ± 2.6%; P < 10⁻⁴), whereas erythroid precursor populations were expanded (12.3% ± 3.3% vs. 3% ± 0.8%; P = 0.002).

To assess whether zebrafish MPD is transplantable, 3 × 10⁶ whole kidney marrow cells were isolated from diseased individuals and introduced into sublethally irradiated recipient fish. In total, five of six MPDs were transplantable with 14 of 26 primary recipients developing MPD by 2 months after transplantation (SI Table 4). Of those MPDs that were transplantable, secondary transplantation resulted in a striking reduction in transplant efficiency (n = 1 of 26 developed MPD; SI Table 4). Taken together, these data suggest that RAS activation in the zebrafish hematopoietic compartment leads to expansion of myeloid cell populations.
Fig. 4. MPD is common in the non-heat-shocked group. (A) Normal zebrafish side view. (B) Double transgenic adult fish (53 dpf) with MPD. (C and G) Hematoxylin–eosin (H&E)-stained sections of normal (C) and MPD kidney (G). (D–F) RNA in situ hybridization of normal kidneys. (H–J) RNA in situ hybridization of MPD kidneys. Antisense probes are designated within each panel. (L) Arrows denote l-plastin-expressing monocytes in normal kidney. (M) Arrowheads denote mpo-expressing granulocytes in normal kidney. (K and M) FACS analysis of whole kidney marrow cells from normal (K) and MPD fish (M). Erythrocytes are shown in red, lymphocytes in blue, granulocytes and monocytes in green, and blood cell precursors in purple. FSC, Forward scatter; SSC, side scatter. (L and N) May–Grunwald–Giemsa-stained cytospins of normal (L) and MPD (N) kidney marrow cells. [Scale bars: 1 cm (A and B), 25 μm (C–J), and 12.5 μm (L and N).]

(26, 27) and ineffective erythropoiesis (28) but does not confer self-renewal potential to progenitor cells.

Intestinal epithelial hyperplasia was also observed in both the heat-shocked and non-heat-shocked groups (36% vs. 16% of histologically examined fish, respectively). In contrast to juvenile fish (day 15–25 dpf; SI Fig. 10), adult fish displaying krasG12D expression in intestinal epithelial cells had more severely disorganized intestinal epithelial architecture, with some foci forming large outgrowths that protrude into the gastrointestinal cavity (SI Fig. 11). The observation that RAS activation in zebrafish intestinal epithelial cells leads to intestinal hyperplasia is consistent with findings that 50% of human colorectal cancers have RAS mutations (29). Additionally, one fish from each treatment group developed an MPNST (SI Fig. 11), which was morphologically similar to those seen in p53 mutant fish (30). These tumors expressed glial fibrillary acidic protein (GFAP) when assessed by in situ hybridization, a finding present in 30% of human MPNSTs.

MPD Can Be Specifically Induced by ex Vivo Heat Shock of Marrow Cells Followed by Transplantation. The zebrafish MPD is of particular interest because the disease is remarkably similar to krasG12D-induced mouse MPD (26, 27), and RAS pathway activation is observed in 30% of human myeloproliferative disorders and leukemia (31). In the heat-shocked group, animals developed MPD with a shorter latency and a lower frequency compared with the non-heat-shocked group, likely because of the severe juvenile lethality and high propensity of animals to develop RMS after heat shock. An ex vivo heat shock strategy was developed to induce krasG12D expression in hematopoietic cells to induce specifically MPD and circumvent RAS-associated lethality and unwanted tumors induced by the early heat shock (Fig. 5A). Double transgenic fish were raised in the absence of heat shock, and hematopoietic cells from the kidney marrow were isolated from healthy double transgenic fish and analyzed by flow cytometric analysis to confirm that myeloid cell expansion was not already present in these animals (Fig. 5B and D). Next, half of the kidney marrow cells were heat shocked ex vivo for 30 min at 37°C, and the other half of the marrow cells were left untreated. Both groups of cells were subsequently transplanted into sublethally irradiated recipient fish. At 2 months after transplantation, 8 of 38 recipient fish from the heat-shocked group had developed MPD (Fig. 5C and D). The other half of the marrow cells were heat shocked ex vivo for 30 min at 37°C, and the other half of the marrow cells were left untreated. Both groups of cells were subsequently transplanted into sublethally irradiated recipient fish. At 2 months after transplantation, 8 of 38 recipient fish from the heat-shocked group had developed MPD (Fig. 5C and D).

Table 1. Differential cell counts of the normal kidneys, spontaneous MPD kidneys, ex vivo heat shock-induced MPD kidneys, and primary recipient kidneys

<table>
<thead>
<tr>
<th></th>
<th>Myelomonocyte</th>
<th>Myelomonocyte</th>
<th>Erythrocyte</th>
<th>Erythroid precursor</th>
<th>Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal kidney (n = 4)</td>
<td>28.8 ± 4.1</td>
<td>6.3 ± 1.3</td>
<td>41.3 ± 2.6</td>
<td>3.0 ± 0.8</td>
<td>20.8 ± 5.6</td>
</tr>
<tr>
<td>Spontaneous MPD kidney (n = 4)</td>
<td>53.8 ± 4.6**</td>
<td>14.8 ± 3.3**</td>
<td>7.3 ± 5.6**</td>
<td>12.3 ± 3.3**</td>
<td>12.0 ± 3.9*</td>
</tr>
<tr>
<td>Ex vivo heat shock MPD kidney (n = 3)</td>
<td>40.7 ± 6.4*</td>
<td>13.3 ± 2.3**</td>
<td>17.0 ± 3.6**</td>
<td>12.7 ± 1.2**</td>
<td>16.3 ± 1.5</td>
</tr>
<tr>
<td>Primary recipient kidney (n = 4)</td>
<td>40.5 ± 5.9*</td>
<td>13.2 ± 2.5**</td>
<td>20.0 ± 5.1**</td>
<td>10.8 ± 2.8**</td>
<td>15.5 ± 2.6</td>
</tr>
</tbody>
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Myelomonocytes include granulocytes, monocytes, and macrophages. Erythroid precursors include cells from proerythroblast to polychromatophilic erythroblast stages. One hundred cells per kidney were analyzed. Numbers are presented as mean ± 1 SD. Statistical significance is indicated by asterisks. *, P < 0.05; **, P < 0.01.
E) whereas no fish receiving untreated marrow cells had disease (0 of 38; \( P = 0.005 \)). MPD arising in these animals was similar to those described above, and no other tumor types were observed (Fig. 5 C and E and Table 1). These results highlight the use of hsp70-Cre and ex vivo heat shock to generate specific tumor types in transgenic animals, even in the absence of tissue-specific gene promoters that drive Cre expression in cell types of interest.

**Discussion**

Inducible Cre/Lox recombination strategies have been widely used in mammalian genetics to conditionally activate gene expression within targeted tissues of interest (32, 33); however, few studies have been reported with Cre/Lox technology in zebrafish (14–16). Here, we describe a transgenic approach where human kRASG12D can be induced by heat-shocked Cre expression in zebrafish. Two ways of using the system were explored, either heating whole live animals or ex vivo heat shock to cells of interest. Using the first approach, we find that heat shock of whole embryos led to increased tumor incidence, earlier tumor onset, and altered tumor spectrum compared with non-heat-shocked animals. These results demonstrate that the heat shock approach is both inducible and amenable to producing various lesions in double transgenic animals. Moreover, ex vivo heat shock of kidney embryos led to increased incidence of MPD in transplanted animals compared with non-heat-shocked controls, providing a robust methodology for inducing specific disease by simply isolating cells from double transgenic animals, ex vivo heat shocking, and transplanting them into irradiated hosts.

Our experiments highlight the use of heat shock-inducible approaches to provide alternatives for conditionally activating genes in whole animals. Compared with other inducible systems, the heat shock-inducible system offers several unique opportunities. First, the recombination can be induced rapidly (4 h after heat shock) and does not require persistent chemical exposure. Second, tissue-specific recombination can be achieved through ex vivo heat shock of cells of interest, a strategy that is especially advantageous in organisms lacking well-characterized promoters or established viral transduction, such as Xenopus, medaka, and zebrafish. Moreover, heat shock can be delivered locally by laser activation of individual cells; thus it may be possible to activate a gene of interest selectively in targeted cell types within the normal microenvironment (34). Because the heat shock response is a fundamental cellular mechanism found in most animals and HS-Cre transgenic mice have been reported (35), this heat shock-inducible Cre/Lox approach could be adapted to other model systems.

Our heat shock-inducible Cre/Lox approach can lead to transgene expression in the absence of inducing stimuli, likely caused by the aberrant expression of the hsp70 promoter (18). However, other inducible approaches used to generate tumor models are also leaky. For example, in a Kras-induced MPD mouse model (27), all double transgenic (LSL-KrasG12D; Mx1-Cre) mice develop MPD regardless of treatment with polynosinic-polycytidylic acid, although prolonged survival was observed in the untreated group (58 days vs. 35 days). In estrogen-responsive transgenic approaches, 23% of CD2-mycER mice develop lymphoma without treatment compared with the incidence of 62% in the tamoxifen-treated group (36). Thus, similar to other approaches, our heat shock-inducible Cre/Lox strategy is also leaky; however, our strategy does not require continuous chemical exposure to induce conditional gene expression, and it can be delivered to a variety of tissue types. Finally, it is likely our approach can be further optimized by using alternative heat shock Cre lines or promoters. Additionally, raising fish at a lower temperature (26.5°C instead of 28.5°C) may result in decreased heat shock promoter activity and lower tumor incidence (H. Feng, D.M.L., J. A. Madge, A. Quinkertz, A. Gutierrez, D.S.N., J. P. Kaneki, and A. T. Look, unpublished data).

To date, we have observed four distinct types of tumors and hyperplasia in this somatic kRASG12D activation RASel: RMS, MPD, intestinal hyperplasia, and MPNST. These RAS-induced zebrafish diseases are morphologically and molecularly similar to those described in human, providing a powerful tool for future chemical and genetic studies for further understanding of RAS-related tumorigenesis. Compared with a “hit-and-run” KrasG12D mouse model (21), in which lung cancer, thymic lymphoma, and papilloma are the most common lesions, the tumor spectrum found in our zebrafish model is different. This finding may be explained partially by the fact that the B-actin promoter used in this work is not ubiquitously expressed and has different levels of expression in different tissue types. More importantly, our findings support that tumor spectrum differs depending on the species and context. For example, mice deficient in p53 develop predominantly lymphomas (37), whereas p53 mutant zebrafish (30) develop malignant peripheral nerve sheath tumors, and the most common malignancy in Li-Fraumeni patients is osteosarcoma (38). Therefore, use of alternative model systems may provide unique opportunities for modeling human disease that are not readily assessable to other cancer models.

Our studies provide the successful use of heat shock-inducible recombination approaches to model cancer in any vertebrate. This heat shock-inducible recombination technology is especially useful in zebrafish but will also be widely applicable to other model organisms. The RAS-induced zebrafish tumor and hyperplasia models generated herein resemble their associated human malignancies, underscoring the utility of this inducible system to aid in our understanding of conserved mechanisms underlying cancer and will be useful for chemical screening for anticancer agents.
Materials and Methods

Animals and Stable Transgenic Lines. Zebrafish were maintained in accordance with Animal Research Guidelines at Children’s Hospital Boston. The B-actin-LoxP-EGFP-LoxP-kRASG12D construct (SI Methods) was linearized with XhoI, purified, and injected into AB-strain zebrafish embryos to generate stable lines. The hsp70-Cre stable transgenic line was generously provided by Andre Quinkertz from Jose Campos-Ortega Laboratory.

Real-Time PCR to Quantify Recombination Efficiency. A real-time PCR (SYBR-Green-I based fluorescence, Bio-Rad, Hercules, CA) assay was developed where the threshold cycle number (Ct) is proportional to the logarithm of the initial amount of template used in the reaction (molecules per reaction). Thus, the number of molecules in an unknown sample can be calculated by comparing with a standard curve generated from samples in which the amount of DNA is known (20). First, a RT-PCR primer set 1 (B-actin forward, 5′-GGCTTATATGGAATATGGAAG; and GFP reverse, 5′-GGTAAACGTCTTCCGCCGTGTC) was designed to amplify a 296-bp fragment that detects the nonrecombined B-actin-lox-EGFP-lox-kRASG12D, whereas primer set 2 (B-actin forward, 5′-GAGTTGACTCCAGATGTCA; and RAS reverse, 5′-CTACGCATCAGTCTAACAT) amplifies a 193-bp fragment found only after recombination (SI Fig. 8A). Next, standard curves were obtained by taking plasmids (B-actin-lox-EGFP-lox-kRASG12D and B-actin-kRASG12D) of known concentration to create a log dilution series for each primer set. A linear titration plot of each primer pair was obtained, and the threshold cycle number (Ct) vs. log of the number of template molecules was generated to correlate the threshold cycle number with the number of template molecules per reaction. A trend line was added and correlated greatly with the individual data points (R² = 0.9918 and 0.997; SI Fig. 8B). Genomic DNA from individual embryos was extracted, and real-time PCR was performed. Recombination efficiency was defined as having >50% of whole kidney marrow cells falling into the myeloid and precursor gates.

Statistical Analysis. Survival curves are presented graphically by using the method of Kaplan and Meier. Differences in death rates were assessed by using both log rank and landmark analysis. Student’s t test was used to compare the recombination efficiency, kRAS-expressing cell number, the body length of fish in different treatment groups, and to compare the total number of whole kidney marrow cells and cell number of different lineages in MPD-affected fish with controls. The Fisher exact test was used to compare MPD incidence in ex vivo heat shock experiments.

DNA in Situ Hybridization and Immunohistochemistry. Antisense and sense control probes were made for human kRASG12D, zebrafish desmin, myoD, myogenin, mpo, and l-plastin, and Cre. Whole-mount RNA in situ hybridization and antibody staining with anti-phospho-ERK1/2 monoclonal antibody (M9692; Sigma, St. Louis, MO) were performed as described previously (39). In situ hybridization and antibody staining on paraffin-embedded sections (GFP antibody JL-8; Invitrogen, Carlsbad, CA) were performed as described previously (10).

Transplantation. Kidney marrow cells were collected and analyzed by FACS analysis as described previously (25). For serial transplantations, 3 × 10⁵ marrow cells from donor fish were introduced into irradiated AB strain adult fish (23 Gy, 2 days before transplantation) by i.p. injection. For ex vivo heat shock transplantations, B-actin-kRASG12D cells (with or without heat shock) with 2 × 10⁶ red blood cells (as carrier cells) from AB strain fish were introduced into irradiated AB strain adult fish (23 Gy, 2 days before transplantation) by intracardiac injection. To assess the engraftment of MPD, kidney marrow cells from recipient fish were analyzed by FACS at 4, 6, or 8 weeks after transplantation. Successful engraftment of MPD was defined as having >50% of whole kidney marrow cells falling into the myeloid and precursor gates.

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